GENETIC ENGINEERING OF ANTHURIUM FOR BACTERIAL DISEASE RESISTANCE

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ABSTRACT

To aid transformation of anthurium, tissue culture and regeneration was achieved through either somatic embryogenesis or shoot regeneration using in vitro grown etiolated internodes, laminae, and root segments. Two gene transfer methods were used to transform anthurium tissues. Using bombardment of DNA-coated microprojectiles into anthurium calli and etiolated internodes, transient expression of ß-glucuronidase (GUS) and neomycin phosphotransferase II (NPTII) was observed in these tissues. No transformed plants were recovered using this method. Antibacterial genes, including an insect attacin gene (*Att*), phage P22 gene *P13*, phage T4 lysozyme gene *e*, and a gene encoding an analog of insect cecropin B (Shiva-1) were driven by either double CaMV35S or potato wound inducible promoter, in the plant expression vector pBI121. *Agrobacterium tumefaciens* LBA4404 carrying either pCa2Att, pCa2P13, pCa2T4, pWIAtt or pWIShiva was used for cocultivation with internode or lamina explants of UH965 and UH1060. Following culture on selection media containing kanamycin and carbenicillin or cefotaxime, shoots regenerated from various calli pieces. Kanamycin-resistant plantlets were recovered from UH965 and UH1060 etiolated internode explants cocultivated with *Agrobacterium* with or without tobacco cell line 'Su' as nurse culture. GUS activity as determined by histochemical staining was absent in the kanamycin-resistant plants evaluated. Western blot analysis of total proteins from lamina calli formed de novo from kanamycin-resistant UH965 plants showed the presence of attacin protein. Polymerase chain reaction was used to amplify DNA fragments from the introduced genes. In six UH965 plants, *Att* and *nptII*

genes were amplified in the expected sizes of 546 and 1054 bp, respectively. In UH1060 plants, *P13* and *nptII* genes were amplified. A GUS gene fragment was amplified in one of the UH965 plants. No amplification of the above-mentioned genes was observed in DNA samples of untransformed control plants. Southern hybridizations using amplified sequences from *Att*, *P13* and *NPTII* all showed positive hybridization. Kanamycin-resistant UH965 plants with Ca2Att, Ca2P13 or Ca2T4 were challenged with the blight pathogen *Xanthomonas campestris* pv. *dieffenbachiae* strain D150 in the petiole end. The result of two challenge experiments indicated that most transgenic UH965 plants were partially resistant and a few were resistant to the blight bacteria.

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CHAPTER 1

INTRODUCTION

Anthurium andraeanum Hort. is generally clonally propagated for cut flower and potted plant production. New cultivars of this outbreeding crop are developed through sexual hybridization and progeny evaluation and selection. Due to the outbreak of a bacterial blight in Hawaii in the early 1980's (Nishijima and Fujiyama, 1985), the anthurium production of Hawaii has steadily declined between 1986 and 1991 (Hawaii Agricultural Statistical Service, 1992) from $10 million to $6.5 million wholesale value. The genetic improvement of anthurium for bacterial disease resistance through biotechnology is an alternative route to the conventional hybridization breeding method.

Blight of anthurium is caused by the bacterium *Xanthomonas campestris* pv. *dieffenbachiae*. The disease was first observed in 1971 on the island of Kauai (Hayward, 1972) with subsequent outbreaks reported on the islands of Oahu and Hawaii in 1981 (Nishijima & Fujiyama, 1985). The bacterium can cause both foliar and systemic symptoms in anthurium. Foliar symptoms include watersoaked and necrotic spots. Systemic, i.e. vascular, infections lead to a general yellowing of entire leaf blades of leaves. Plants with systemic infection in the stem, petioles, and spathes have very high internal bacterial populations and usually die (Nishijima & Fujiyama, 1985). The effects of infection can be intensified by warm and humid conditions. The disease can be spread by various means such as splashing rain or irrigation,

or the use of contaminated cutting tools during cultural operations such as pruning and handling.

Various blight control measures, including limited use of antibiotics, were suggested previously. The most effective one at the present time is strict sanitation together with integrated control measures including a clean stocks program. Despite implementation of control efforts by many growers, annual anthurium production has declined steadily.

One reliable approach for the control of anthurium blight is the cultivation of resistant varieties. Genetic resistance combined with other control measures would provide an effective line of defense against anthurium blight. For this purpose, it is desirable to transfer the apparent systemic resistance from *Anthurium antioquiense* to the cultivated *A. andraeanum* (Kamemoto et al., 1990). However, due to the small flower of *A. antioquiense*, the F1 hybrids have to be backcrossed to the cultivated varieties in order to obtain resistant plants with horticulturally desirable characteristics. This process could take eight or more years to achieve the goal, due to the generations of sexual crossing involved and the long life cycle of anthurium (3 years from seed to seed). More importantly, the quality of the final resistance may not be as high as desirable.

The mode of inheritance of the available resistance in the breeding germplasm remains to be determined.

Methods for crop improvement through biotechnologies have been evolving in the last few years. Use of restriction fragment length polymorphisms (RFLPs) has been proposed to facilitate the identification of resistance genes against bacteria (*Pseudomonas*) (Martin et al., 1991), tobacco mosaic virus (Tanksley et al., 1989), or root-knot nematode (Aarts

et al., 1991; Klein-Lankhorst et al., 1991) in tomato. Such methodology is not currently useful for anthurium breeding, due to the long life cycle, and the lack of understanding of the genetic basis of blight resistance. Therefore, it would be desirable to adopt alternative approaches which allow for incorporation of resistance genes from sources other than anthurium into cultivars currently in production.

One recent advance to this end has been the identification in insects and some mammalian species of several peptides with antibacterial properties (Boman, 1991; Ganz et al., 1990). These novel types of peptides work independently or in concert to kill a range of important Gram-positive and -negative bacteria. Natural or synthetic insect antibacterial genes have been cloned, modified and introduced for expression in plants such as potato (Destéfano-Beltrán et al., 1990).

The overall goal of this research is the introduction of various antibacterial genes from *Hyalophora cecropia* and bacteriophages, in suitable vectors, into anthurium cultivars followed by plant characterization and evaluation for bacterial resistance. To introduce foreign genes into anthurium, a reliable regeneration system under antibiotic selection conditions has to be developed. In Chapter 2, I review the general aspects of anthurium, its breeding, the bacterial blight, the source of antibacterial genes, aspects of introducing genes into plants, and progress in tissue culture and regeneration of anthurium and other aroids. Chapter 3 describes experiments on tissue culture and regeneration of anthurium through organogenesis and somatic embryogenesis, and their potential application for genetic transformation. Chapter 4 describes the use of *Agrobacterium*-mediated transformation system to obtain transgenic anthurium, and the

characterization of transgenic anthurium. Resistance of transgenic anthurium to the bacterial pathogen *X. campestris* pv. *dieffenbachiae* was tested by challenge-inoculation with a virulent strain of this bacterium. Chapter 5 describes the results of microprojectile bombardment-mediated gene transfer with anthurium. Appendices describe cocultivation of anthurium etiolated internodes with *Agrobacterium* carrying antibacterial genes with signal sequences, cocultivation of tobacco leaf explants with *Agrobacterium*, and protocols for cocultivation of anthurium and tobacco explants.

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CHAPTER 2

LITERATURE REVIEW

2.1 ANTHURIUM

Anthurium is the largest and most complex genus in the family Araceae. It consists of about 1000 species (Croat, 1992). The distribution of this genus extends from Northern Mexico and the Greater Antilles to Southern Brazil and Northern Argentina and Paraguay (Croat, 1983, 1986). *Anthurium* was originally placed under the subfamily Pothoideae by Croat (1983) and Grayum (1990). Bogner & Nicolson (1991) removed the genus from the subfamily Pothoideae and reclassified the subfamily Lasioideae.

Anthurium plants possess very small hermaphroditic (bisexual) flowers, closely arranged in a series of spirals on the spadix (Bogner & Nicolson, 1991; Croat & Bunting, 1978; Croat, 1980; Grayum, 1990; Higaki et al., 1984). The four stamens are enclosed in four fleshy perianth segments called tepals. The ovary is hypogynous with two fused carpels. The ovary has two locules, and one or two ovules per locule (Higaki et al., 1984; Bogner & Nicolson, 1991). One feature of anthurium is protogyny, which means the stigma matures earlier than stamen (Croat, 1980; Higaki et al., 1984). Usually stigmatic droplets are produced when the stigma becomes receptive. In *A. seibertii* Croat, the stigmatic fluid contained 8% sugar, composed of sucrose, glucose and fructose (Croat, 1980). The commercial flower is a combination of a colorful modified leaf, known as the spathe,

and the spadix. The inflorescence emerges about a month after leaves appear and precedes the next new leaf by a few weeks (Higaki et al., 1984). Leaves are simple with netted veins (Croat & Bunting, 1978).

The chromosome number of most investigated species is 2n = 30. There are four polyploid series: 20-40-60, 24-30-48-84, 28-56, and 30-60-90-ca 124 (Sheffer & Kamemoto, 1976; Sheffer & Croat, 1983). The chromosome number of the miniature, lavender-flowered *A. amnicola* Dressier is 30. This tulip-type anthurium was used extensively in the breeding of novel cultivars due to its crossability to other important anthurium species, such as *A. andraeanum* (2n = 30) (Marutani et al., 1988).

Commercial production of anthurium has been focused on two major species: *A. andraeanum* and *A. scherzerianum*. *A. andraeanum* is grown mostly for cut flower production. Main production areas are Hawaii, the Netherlands, and some other tropical and subtropical countries. *A. scherzerianum* is sold as a flowering potted plant. The main production areas are western and central European countries (Geier, 1990).

2.1.1 Bacterial blight and its current control

Bacterial blight of anthurium in Hawaii was first observed on the island of Kauai in August 1971 on the cultivar 'Kansako Red' (Hayward, 1972). The disease was later found on many aroids grown on all Hawaiian islands (Nishijima & Fujiyama, 1985). The disease is caused by *Xanthomonas campestris* pv. *dieffenbachiae* (McCulloch & Pirone) Dye (*Xcd*). The bacterium infects other aroids in addition to *Anthurium*: *Aglaonema*, *Dieffenbachia*, *Philodendron*, *Syngonium*, *Epiprenum*, *Colocasia*, *Alocasia*,

Spathiphyllum, *Rhaphidophora*, *Caladium* and *Xanthosoma* (Hayward, 1972; Lipp, 1988; Nishijima, 1988; Norman & Alvarez, 1989).

The symptoms caused by the bacteria include foliar watersoaked spots near the leaf margin and chlorosis. Systemic infection can occur through vascular system and spread throughout the plant very rapidly (Nishijima, 1988). Current control measures include strict sanitation to prevent the buildup of bacterial inoculum, and preventing the spread of the bacterium by splashing rain, irrigation, tools and other means by disinfestation of tools and other techniques (Nishijima, 1988). Although the antibiotic combination of streptomycin and oxytetracycline has been used in some cases, the bacteria developed streptomycin resistance in a short time. Therefore the antibiotics are not recommended for routine control (Alvarez et al., 1989; Nishijima, 1988). Studies using several chemicals, such as Physan (Maril Products, Tustin, California) (Alvarez et al., 1990) and fosetyl aluminum, commercially named as Aliette 80 WP (Rhône-Poulenc Agrochimie, Lyon, France), have indicated some degree of control over the bacterial infection (Alvarez et al., 1991).

2.1.2 Breeding for disease resistance

Anthurium was first introduced into Hawaii in 1889. It became popular among hobbyists during the 1940's. The breeding program was initiated in 1950 by Dr. H. Kamemoto. Early work on selection and hybridization of many accessions yielded the first University cultivars 'Uniwai', 'Marian Seefurth', and 'Mauna Kea'. Thereafter many new germplasms were added

to the existing breeding program for evaluation of disease resistance and horticultural characters (Kamemoto et al., 1986).

The major problems in production of anthurium are anthracnose, burrowing nematode and bacterial blight. Evaluation of accessions for anthracnose (*Colletotrichum gloeosporioides*) resistance have shown a wide variation in resistance and susceptibility. Some cultivars, such as 'Marian Seefurth', 'Manoa Mist', 'Abe', 'Anuenue', and 'Mauna Kea', etc (Aragaki et al., 1968; Higaki et al., 1979) are resistant. Anthurium decline caused by the burrowing nematode, *Radopholus similis*, can be controlled by applications of a nematicide (Kamemoto, 1988). The new outbreak of bacterial blight since 1980 has become a major problem in the production of anthurium cut flower in Hawaii. Most commercial cultivars are susceptible to the disease infection. Blight resistant cultivars are necessary in addition to every possible control measure. An intense breeding program was initiated, using the blight resistant species *A. antioquiense* as one parent and other susceptible commercial cultivars, such as 'Marian Seefurth' and 'Paradise Pink' as another parent. F1 hybrids had a high degree of resistance to the bacteria. However, they were not acceptable in terms of horticultural characteristics. Therefore, backcrossing is necessary to improve the flower (Kamemoto et al., 1990; 1991). Some cultivars from the Univ. of Hawaii do show tolerance to the blight: 'Blushing Bride', 'Anuenue', 'Kalapana' and 'Tropic Ice' (Kamemoto et al., 1991). Despite these breeding efforts, the genetic basis of blight resistance is still not clear. Therefore, an alternative breeding program was initiated by using genetic transformation of anthurium with antibacterial genes of non-plant sources (Kuehnle, 1989).

2.2. GENETIC ENGINEERING FOR DISEASE RESISTANCE

Genetic engineering techniques have been successfully utilized for the introduction of pest resistance into plants using genes which are of non-plant origin, i.e. bacterial or viral. These studies include insect resistance in transgenic tobacco (Barton et al., 1987; Perlak et al., 1991; Vaeck et al., 1987), tomato (Perlak et al., 1991; Fischhoff et al., 1987; Delannay et al., 1989), and cotton (Perlak et al., 1990), using a toxin gene from *Bacillus thuringiensis*; tobacco hornworm insect resistance in transgenic tobacco using cowpea trypsin inhibitor gene (Hilder et al., 1989a, b); fungal disease resistance in tobacco using ribosome-inactivating protein (Logemann et al., 1992) or bacterial chitinase (Jach et al., 1992), and virus resistance in various crops using viral coat or capsid protein gene (Beachy et al., 1990; Fitch et al., 1992; Gielen et al., 1991; Jongedijk et al., 1992; Ling et al., 1991; Quemada et al., 1991) or replicase genes (Anderson et al., 1992; Braun & Hemenway, 1992; Carr & Zaitlin, 1991).

Research on the engineering of plants for bacterial disease resistance is being pursued in several crops only in recent years. Jaynes et al. (1987, 1992) suggested that some antimicrobial genes encoding lytic peptides from insects, chicken lysozyme, and phage lysozymes, can be engineered to increase bacterial disease resistance in plants. Destéfano-Beltrán et al. (1990a) and Nagpala et al. (1990) have introduced several natural (attacin, chicken lysozyme, T4 phage lysozyme, P22 phage genes *13* and *19*, and cecropin B) or synthetic (Shiva-1, Shiva-3, Shiva-4 and Shiva-30) antibacterial genes into tobacco and potato. Southern blot analysis confirmed the integration of introduced genes into the plant genome.

Transgenic potatoes carrying a cecropin B derivative, SB-37, are being evaluated for resistance to bacterial pathogen, *Pseudomonas solanacearum* (Destéfano-Beltrán et al., 1990b). Bacteriophage T4 lysozyme gene with a plant α-amylase signal peptide has also been introduced into potato with the hope of providing resistance against *Erwinia carotovora* (Düring, 1991a). Transgenic tobacco producing T4 lysozyme was also obtained using the same construct (Düring, 1991b; Düring & Hippe, 1989; Hippe et al., 1989).

Nordeen et al. (1992a) determined the lethal concentration of a cecropin derivative SB37 (Jaynes et al., 1988) for different pathobacteria, such as *Pseudomonas syringae* pv. *glycinea* strain Pg4 and *P. syringae* pv. *tomato* strain 0683-23. The lethal concentrations ranged from 0.1 to 4.5 μM using a thin-agarose plate assay. *Xanthomonas campestris* pv. *vesicatoria*, another pathovar of *X. campestris*, was killed at 0.6 μM. A gene for a cecropin derivative was ligated to a barley α-amylase secretory sequence (the resulting gene was named MB39) and introduced into tobacco. Northern blot analysis indicated the expression of the cecropin gene (Nordeen & Owens, 1992; Nordeen et al., 1992b). Montanelli and Nascari (1991) used *A. rhizogenes* R1000 to introduce the same cecropin gene, but driven by a double 35S promoter, into potato. When crude leaf extract of transgenic potato was assayed for antibacterial activity, an inhibition zone was detected against *P. solanacearum* race 3. The potato soft rot bacterium *E. carotovora* was killed by cecropin and chicken lysozyme at 6-8 μg/ml (Sinden et al., 1992). A test for resistance to soft rot indicated only minor differences among the tubers from control potato plants and putative transformants (Hassan et al., 1992). A similar approach using cecropin B in peach is being investigated (Mills & Hammerschlag, 1992). Recently, an

active form of hen egg white lysozyme (HEWL) gene has been successfully introduced into tobacco. Transgenic tobacco plants expressing HEWL were active against *Micrcococcus luteus* (Trudel et al., 1992).

From the above discussion, it appears that introduction of antibacterial genes into plants to confer bacterial disease resistance is potentially applicable for anthurium. Souces of antibacterial proteins are described in the following sections.

2.2.1 Insect immune proteins

The insect immune proteins, or antibacterial peptides (Table 2-1), were initially isolated and characterized in some detail by Boman's group in Sweden (Boman, 1991; Boman & Steiner, 1981; Boman & Hultmark, 1987; Boman et al., 1991). Other groups have also isolated similar immune proteins from different insects (Table 2-1). These peptides are the products of humoral immunity in insects, such as the giant silk moth *Hyalophora cecropia*, after infection by bacteria. Some insects such as *Phormia terranovae* (Lambert et al., 1989; Hoffmann & Hetru, 1992; Reichhart et al., 1992b; Table 2-1) also produce defensin-like peptides. Another type of antibacterial protein, diptericin, has been isolated from fruit fly (Reichhart et al., 1992a; Wicker et al., 1990).

Three classes of bactericidal proteins with potent antibacterial activity have been identified in the giant silk moth: lysozymes, cecropins, and

Table 2-1. Antibacterial peptides isolated from different animals[a]

Species	Life stage/ tissue[b]	Defensive protein	MW (kDa)	Biological activity	Reference
Insects					
Hyalophora cecropia (giant silkmoth)	P	Lysozyme	15	Muramidase	Boman & Steiner 1981
		Cecropins	4	Bactericidal	von Hoffsten et al. 1985
		Attacins	22	Bacteriostatic	Engström et al. 1984; Kockum et al. 1984
		P4	48	Unknown	Lee et al. 1983
Sarcophaga peregrina (flesh fly)	L	Sarcotoxins I	4	Bactericidal	Okada & Natori 1985
		Sarcotoxins II	26	Unknown	Ando et al. 1987; Ando & Natori 1988
		Sarcotoxins III	7	Bactericidal	
	CL	Sapecin	4	Bactericidal	Matsuyama & Natori 1988
Phormia terranovae (blowfly)	L	Diptericins	9	Bactericidal	Dimarcq et al. 1988, 1990
		Defensins	4	Bactericidal	Lambert et al. 1989
		Cecropin-like peptides	4	Bactericidal	
Manduca sexta (tobacco hornworm)	L	Lysozyme	14	Muramidase	
		Cecropin-like peptides	4	Bactericidal	Dickinson et al. 1988
		Attacin-like proteins	22	Bactericidal	Dickinson et al. 1988
		P4-like proteins	48	Unknown	Dickinson et al. 1988
Drosophila melanogaster (fruit fly)	A	Cecropins	4	Bactericidal?	Kylsten et al. 1990
	GT	Andropin	6	Bactericidal?	Samakovlis et al. 1991
	A	Diptericin	9	Bactericidal	Wicker et al. 1990

Table 2-1. (continued) Antibacterial peptides isolated from different animals[a]

Species	Life stage/ tissue[b]	Defensive protein	MW (kDa)	Biological activity	Reference
Apis mellifera (honeybees)	A	Apidaecins	2	Bacteriostatic	Casteels et al. 1989
		Abaecins	4	Bactericidal?	Casteels et al. 1990
Zophobas atratus (tenebrionid beetle)	L	Coleoptericin	8	Bactericidal	Bulet et al. 1991
Animals					
Homo sapiens	CL	Defensin	5	Bactericidal	Daher et al. 1988
	PMN	BPI	55-60	Bactericidal/PI[c]	Lehrer & Ganz 1990
Rabbit	M	Defensins	4	Bactericidal	Ganz et al. 1989
	G	NPs	4	Bactericidal/PI	Selsted et al. 1984
Pig	I	cecropin P1	3.3	Bactericidal?	Lee et al. 1989
	TM	TAP	3.4-4.0	Bactericidal?	Diamond et al. 1991
	BM	BPI	25	Bactericidal	Leong & Camerato 1990
	N	Indolicidin	1.4	Bactericidal	Selsted et al. 1992
Rat	M	Defensins	3.2-3.8	Bactericidal	Eisenhauer et al. 1989.
Mouse	I	Cryptdins	4	Bactericidal?	Selsted et al. 1992
Guinea pig	Lc	GNCPs	2.6	Bactericidal/PI	Nagaoka et al. 1992
Xenopus laevis (African clawed frog)	S	Magainins	2-3	Bacteriostatic?	Zasloff 1987
	St	PGQ	1.4-2	Bacteriostatic?	Moore et al. 1991
Bombina variegata	S	Bombinin-like peptides	3	Bactericidal	Simmaco et al. 1991

Table 2-1. (continued) Antibacterial peptides isolated from different animals[a]

Species	Life stage/ tissue[b]	Defensive protein	MW (kDa)	Biological activity	Reference
Tachypleus tridentatus (horsehoe crab)	H	Tachyplesins	2	Bactericidal/ permeability	Nakamura et al. 1988 Ohta et al. 1992

[a] Partly from Dunn (1990, 1991)

[b] A, Adult; BM, Bone marrow; CL, Cell line; G, Granulocytes; GT, Genital tracts; H, Hemocytes; I, Intestine; L, Larva; Lc, Leucocytes; M, Macrophages; P, Pupa; PMN, Polymorphonuclear neutrophils; S, Skin; St, Stomach; TM, Trachael mucosa

[c] PI, Permeability-increasing

attacins (Hultmark et al., 1980). The cecropia lysozyme is very similar to chicken egg white lysozyme in enzyme activity (Boman et al., 1985). Six different forms of attacins, with an apparent molecular weight of about 20 kDa, are the largest antibacterial peptides found in the hemolymph of immunized cecropia pupae, and can be distinguished based on isoelectric point (Hultmark et al., 1983). There are three major forms of cecropins. These cecropins are basic and small, around 4 kDa. They are the most potent group of the antibacterial factors, with a broad spectrum of antibacterial activity against both Gram-negative and Gram-positive bacteria (Jaynes et al., 1987).

The mechanism of action of some antibacterial peptides have been studied. Cecropins can lyse both Gram-negative and Gram-positive bacteria (and liposomes) but not eukaryotic cells. The primary targets of these lytic peptides are the inner and outer bacterial membrane (Boman, 1991). Recently, Carlsson et al. (1991) showed that attacin inhibits the synthesis of outer membrane proteins in *E. coli* by interfering with transcription. Lysozymes can cleave the glycosidic bond between the C-1 of N-acetylmuramic acid and the C-4 of N-acetylglucosamine in the bacterial peptidoglycan (Jollès and Jollès, 1984).

2.2.2 Defense proteins from other animals

Other defense proteins or antibacterial peptides have recently been isolated from diverse animal tissues (Table 2-1). Mammalian cells or tissues contain one group of proteins called defensins (Ganz et al., 1990), which are small basic molecules of 29-34 amino acid residues, and have low molecular

weight (about 3 to 5 kDa). They kill a wide variety of gram-positive and gram-negative bacteria and fungi. The primary targets of defensins are the inner and outer membranes of bacteria. They also form ion channels in artificial membranes (Boman, 1991; Lehrer et al., 1991). Some examples are human defensins, rabbit defensins, and rat defensins (Table 2-1). Recently, Selsted et al. (1992) reported a novel bactericidal peptide (indolicidin) with only 13 amino acid residues isolated from the cytoplasmic granules of bovine neutrophils. Both a Gram-positive and a Gram-negative bacteria were killed in 2 hours by the peptide at low concentrations (2.5-10 μg/ml).

Mammals also produce cecropin-like peptides with molecular weight similar to those peptides found in insects. The cecropin P1 was isolated from pig intestine. It has a molecular weight of 3.3 kDa (Lee et al., 1989).

Another group of defense proteins from mammals includes bactericidal/permeability increasing factor (BPI) proteins (Table 2-1). BPI proteins are bactericidal for some Gram-negative bacteria with defective or short-chain lipopolysaccharide (LPS). The cDNA of human BPI has been cloned and showed homology to a LPS-binding protein synthesized by human hepatocytes (Lehrer & Ganz, 1990). BPI from pig is smaller than human BPI. It has a molecular weight of 25 kDa (Leong & Camerato, 1990).

Amphibian skin contains a high percentage of antimicrobial factors. Magainins are one group of small peptides (23 amino acid residues) isolated from the African clawed frog, *Xenopus laevis* (Zasloff, 1987; Berkowitz et al., 1990). Magainins have broad spectrum activity on Gram-negative and Gram-positive bacteria, fungi and parasites (Berkowitz et al., 1990).

2.2.3 Lysozymes

Lysozymes are muramidases cleaving the glycosidic bond in the bacterial peptidoglycan. The most intensively studied lysozymes are those from avian egg-white. There are two types of enzyme: hen egg-white lysozyme or chicken type (c-type), and goose egg-white lysozyme (g-type). The complete primary structures have been elucidated for many lysozymes . Mammalian, insect and invertebrate tissues also contain lysozymes. The molecular weight of these lysozymes range from 14 kDa to about 25kDa. Different groups of lysozymes have different substrate specificity (Jollès & Jollès, 1984). The g-type lysozyme was cloned, sequenced, and compared to c-type lysozyme. No homology was found between the two types of lysozymes (Nakano & Graf, 1991). A human lysozyme gene has also been cloned, modified and expressed in heterologous systems (Omura et al., 1992).

Many bacteriophage lysozyme genes and their protein products have been isolated, cloned (Table 2-2), and sequenced. Usually 2 or 3 lytic proteins are involved in the lytic cycle of bacteriophage. These proteins lyse the bacterial host which results in the release of new phage particles. The phage lambda of *E. coli* contains *R*, *Rz* and *S* genes (Table 2-2). The *R* gene product is a transglycosidase which can degrade the periplasmic peptidoglycan layer (Bienkowska-Szewczyk et al., 1981). The *R* gene has been cloned in expression vectors (van de Guchte et al., 1992; Soumillion & Fastrez, 1992). The *S* gene, which produces an enzyme with pore formation activity, has been cloned and expressed in yeast (Garrett et al., 1990).

Table 2-2. Properties of some phage lysis proteins

Phage	Gene	MW (kDa)	Function	Reference
Lambda	*R*	17.5	Transglycosidase	Bienkowska-Szewczyk et al. 1981
	S	8-9	Pore formation	Garrett et al. 1990
	Rz	?	Endopeptidase?	Young et al. 1979; Casjens et al. 1989
P22	*13*	11.5	Pore formation	Rennell & Poteete 1985
	19	16	Lysozyme	Rao & Burma 1971; Rennell & Poteete 1985
	15	16	Endopeptidase?	Casjens et al. 1989
Cp-1	*cpl-1*	39	Lysozyme	Garcia et al. 1987
ϕ29	*14*	15	Lysozyme?	Garvey et al. 1986
	15	28	Lysozyme	Saedi et al. 1987
ϕ6	*P5*	24	Endopeptidase	Caldentey & Bamford 1992
LL-H	*lysin*	54, 75	Lysozyme	Trautwetter et al. 1986
T4	*e*	18.7	Lysozyme	Tsugita et al. 1968; Weaver et al. 1985
T7	*3-5*	16.8	Lysozyme (amidase)	Dunn & Studier 1983

Antibody against the S-ß-galactosidase fusion protein is available for detection of S protein induction in *E. coli* (Zagotta & Wilson, 1990).

The *19* gene of phage P22 of *Salmonella typhimurium* encodes a protein with enzymatic function similar to *R* gene, but *19* and *R* lack sequence homology (Renell & Poteete, 1985). The *13* gene product of P22 phage is a protein with pore formation activity. The proteins 13 and S share 89% amino acid sequence homology. *Rz* and *15* genes encode proteins that share 59% sequence homology. They have endopeptidase-like activity (Renell & Poteete, 1985; Casjens et al., 1989).

Lysozyme of *E. coli* T7 phage is an N-acetylmuramyl-L-alanine amidase (Inouye et al., 1973). Its gene (*3.5*) sequence has been determined (Dunn & Studier, 1983) and cloned in an expression vector (Moffatt & Studier, 1987). T4 lysozyme encoded by the *e* gene has been actively used in crystallographic and NMR studies (Faber & Matthews, 1990; Lu & Dahlquist, 1992). The *e* gene has been sequenced (Owens et al., 1983) and cloned in various expression vectors (Muchmore et al., 1989; Perry et al., 1985; van de Guchte et al., 1992). Tobacco plants transgenic for T4 lysozyme have been generated recently (Hippe et al., 1989).

The lytic enzyme P5 of phage of *Pseudomonas syringae* pv. *phaseolicola*, ϕ6, has been characterized biochemically (Caldentey & Bamford, 1992). The molecular weight of P5 is 24 kDa. P5 protein is active against several Gram-negative bacteria, including phytopathogenic *P. syringae* pv. *phaseolicola* strain HB10Y (Caldentey & Bamford, 1992). The protein is shown to be an endopeptidase cleaving the peptide bridge between meso-diaminopimelic acid and D-alanine (Caldentey & Bamford,

1992). The nucleotide sequence of *P5* gene has been determined (McGraw et al., 1986).

All the above lysozymes or lytic proteins are produced from phages for Gram-negative bacteria. Bacteriophages from Gram-positive bacteria also produce lysozymes. Some lysozyme genes have been cloned, such as *Bacillus subtilis* phage ϕ29 genes *15* and *28* (Garvey et al., 1986; Saedi et al., 1987), *Streptococcus pneumoniae* phage CPL-1 gene cpl-1 (García et al., 1987; Sanz & García, 1990), and *Lactococcus lactis* LL23 phage LL-H lysozyme lysin (Trautwetter et al., 1986) (Table 2-2). A detailed review of bacterial lysis has recently been published (Young, 1992).

2.3. GENETIC TRANSFORMATION

Several techniques have been devised for the transformation of plant cells or tissues. Subsequent recovery of transformed plants depends on reliable methods of tissue culture and regeneration of plants, generally under selective conditions. Most of the transgenic plants obtained are through *Agrobacterium*-mediated transformation (Klee & Rogers, 1989). Other methods include virus vector (Agroinfection) and direct gene transfer. In recent years, the invention of the particle gun or biolistic process (Sanford et al., 1987) has made possible the transfer of genes to recalcitrant plant species.

2.3.1 *Agrobacterium*-mediated transformation

The soil bacterium *Agrobacterium tumefaciens* causes crown gall in many dicots and some monocots (De Cleene, 1985; De Cleene & De Ley, 1976). The bacterium can naturally transfer a segment of DNA, the T-DNA, from a large tumor-inducing (Ti) plasmid to the plant cell. The infected tissue later forms a tumor (Kado, 1991; Zambryski, 1989). Another species *A. rhizogenes* induces 'hairy' root formation upon infection of many dicots and some monocots (Porter, 1991). *A. rhizogenes* contains a root inducing (Ri) plasmid (Petersen et al., 1989) with similar structure to the Ti plasmid. The hormone genes in the T-DNA are responsible for tumor formation, called neoplasia. Two of the genes encode enzymes for auxin biosynthesis in the gall: *iaaM* (gene *1* or *tms1*) and *iaaH* (gene *2* or *tms2*), which encode tryptophan monooxygenase and indoleacetamide hydrolase, respectively (Schröder et al., 1984; Thomashow et al., 1984). The third gene in this region is *ipt* (gene *4* or *tmr*), which encodes isopentenyl transferase. It catalyses the formation of a cytokinin, isopentenyl adenosine monophosphate (Akiyoshi et al., 1984). Since the synthesis of auxin and cytokinin leads to tumor proliferation, which is not desired for genetic engineering, the hormone genes are usually deleted from the T-DNA. The resulting plasmid is called a "disarmed" Ti plasmid, such as the one in *A. tumefaciens* LBA4404 (Ooms et al., 1981).

T-DNA can be divided into several regions which are essential for plant transformation. One region is the border sequences. The T-DNA is bordered by imperfect 25 base pair repeats (Wang et al., 1984). The right border sequence is essential for integration of T-DNA into the plant chromosome

(Shaw et al., 1984). A second component essential for transformation is the *vir* region. In the nopaline-type Ti plasmid, there are six *vir* loci or operons, *virA*, *virB*, *virC*, *virD*, *virE*, *virG* (Kado, 1991; Zambryski, 1992). The gene products are thought to be involved in T-DNA processing and transfer. The product of *virA* probably is an acetosyringone (AS) sensor. AS is a phenolic compound released from wounded plant tissues, and acts as inducer of the *vir* region. *virG* encodes a *vir* transcriptional regulator. *virE* encodes single-strand DNA-binding proteins. *virE2* fused to reporter *gus* gene localizes to plant cell nuclei. When transgenic plants producing VirE2 protein were inoculated with an avirulent *Agrobacterium virE2* mutant, tumorigenicity was restored. This further supports the hypothesis that the function of VirE2 is to help the T-DNA locate the plant cell nucleus (Citovsky et al., 1992). *The product of virB* is involved in T-DNA transfer structure. *virD* locus can be divided into 2 genes: *virD1* encodes a protein with DNA-relaxing activity, and *virD2* encodes a strand-specific and sequence-specific endonuclease. An experiment using a *virD2* and *lacZ* gene fusion expressed in transgenic tobacco has indicated that VirD2 protein can act as a plant nuclear targeting signal (Herrera-Estrella et al., 1990). The nuclear targeting signal of VirD2 consists of two regions containing 4-5 basic amino acids located within 34 amino acids of the C-terminal (Howard et al., 1992).

The function of *virC* is not clear (Zambryski, 1989). It encodes two proteins VirC1 and VirC2, which have been shown to enhance T-DNA border nicking in *Agrobacterium* (Toro et al., 1988). Recently, the entire *vir* region of the plasmid pTiC58 has been completely sequenced, and two more open reading frames were identified (Rogowsky et al., 1990). This will help further characterization of the structure and function of the *vir* regulon.

Detailed genetic and chemical studies from several laboratories have revealed that the virulence function of the Ti plasmid is induced by some plant phenolic compounds, including acetosyringone (AS) (Stachel et al., 1985;). Other phenolic compounds have been elucidated as *vir* inducing factors. Ashby et al. (1988) reported that the following phenolics act as *vir*-inducer in *A. tumefaciens* C58C: AS, sinapic acid, syringic acid, vanillin, ferulic acid and 3,4-dihydroxybenzoic acid. Bolton et al. (1986) was able to induce *virE* locus using seven phenolics: catechol, gallic acid, pyrogallic acid, p-hydroxybenzoic acid, protocatechuic acid, ß-resorcylic acid, and vanillin. These phenolics are known to be components in the intact and wounded plant cells. Delmotte et al. (1991) showed that acetosyringonyl ß-fucopyranoside, As, acetovanillone, syringaldehyde and syringic acid were able to induce the VirE-Z fusion protein (Z is the protein product, ß-galactosidase, of bacterial *lacZ*). Hess et al. (1991) reported that the aglycone of the dihydrodiconiferyl alcohol glycosides can induce *virE-lacZ* expression. They showed that the inductive phenolics must be constantly present to maintain expression of the *vir* region.

A compound, ethyl ferulate, isolated from wheat cell suspension culture was able to induce *vir* activity. Wheat (*Triticum monococcum*) is a monocot. Extract of asparagus, also a monocot, was able to induce *vir* activity but to a lesser degree than ethyl ferulate (Messens et al., 1990).

Two types of transformation vectors have been developed. One is cointegrative vector which, by itself, cannot replicate in *Agrobacterium*. These vectors are maintained only by cointegrating into an endogenous plasmid, usually Ti plasmid. The donor plasmid contains genes of interest and a sequence of homology to the Ti plasmid. Upon introduction into

Agrobacterium they can recombine with the Ti plasmid to form a cointegrate. Two cointegrating plasmids are available at present. One is based on the disarmed Ti plasmid pGV3850 (Zambryski et al., 1983), the other one is so called split-end vector (SEV), in which the right border and all of the phytohormone genes have been deleted from the Ti plasmid pGV3111 (Fraley et al., 1985).

Another type of the transformation vector is the binary vector, which contains broad-host-range plasmid replication origins (*oriV*) and border sequences. Binary vectors can replicate autonomously in an *Agrobacterium* host carrying a disarmed or armed Ti plasmid. Many binary vectors are available. One of the most frequently used is pBI121 (Jefferson et al. 1987), a derivative of pBIN19 (Bevan, 1984). Other binary vectors with multiple cloning sites were also developed, such as pMON505, pMON530 (Rogers et al., 1987), pGA482, pGA492, pGA580 (An, 1987). A new binary vector (pCIT20) with a multiple cloning site inserted in the *lacZ* gene was developed recently (Ma et al., 1992). The inserted DNA fragment can be screened with the blue-white color test using X-Gal as the substrate. It also contains a selectable marker gene encoding hygromycin phosphotransferase (*Hph*) for selection of transformed plant cells (Ma et al., 1992). Transformation/cosmid vectors are also developed (pCIT30, pCIT101, pCIT103) for cloning plant genes from a cosmid library and subsequent introduction into plant cells without further subcloning (Ma et al., 1992).

A selectable marker is very important for the effective selection of transformed plant cells. The selective agent must be inhibitory to the growth of nontransformed, i.e. wild type, cells. At the same time, the transformed cells must be able to grow and divide under selection conditions. Several

selectable markers have been used in plant transformation, including chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase II (NPTII), hygromycin phosphotransferase (HPH), dihydrofolate reductase, and enzymes for overcoming herbicides (Klee & Rogers, 1989). The most widely used selectable marker is the bacterial NPTII, which confers resistance to some aminoglycoside antibiotics such as kanamycin and G418 (Beck et al., 1982; Bevan et al., 1983; Klee & Rogers, 1989). A *cat* gene is used in a transformation vector (Herrera-Estrella et al., 1983). Both *nptII* and *cat* genes are fused to the promoter of the nopaline synthase gene from *Agrobacterium*. Hygromycin can be used in selection of transformed plant cells for which other antibiotics are not effective. The *Hph* gene encoding hygromycin phosphotransferase has been used in various vectors, such as pCIT20 (Ma et al., 1992).

Another useful marker in the transformation vector is a reporter gene, whose product can be scored or visualized after enzymatic reaction in transformed cells or tissues. Genes encoding ß-galactosidase, ß-glucuronidase, and luciferase, respectively, have been widely used as reporters. The *uidA* (*gus*) gene encoding ß-glucuronidase (GUS) was cloned from *E. coli* (Jefferson, 1989; Jefferson et al., 1986). Many ß-glucuronides can be used as substrates for the GUS enzyme, such as the fluorometric substrate 4-methyl-umbelliferyl-glucuronide (4-MUG) and the chromogenic substrate 5-bromo-4-chloro-3-indolyl-ß-glucuronide (X-gluc). The measured fluorescence produced upon cleavage of 4-MUG by GUS indicates the level of GUS activity. X-gluc can be cleaved by GUS to form a blue precipitate in a reaction mix; the blue color can be visualized and scored. Firefly luciferase (LUC) (de Wet et al., 1987) expressed in plant cells or protoplasts has been

very useful for measuring the level of transient and stable expression (Ow et al., 1986). Bacterial luciferase (LUX) has been used for the same purpose (Koncz et al., 1987, 1990; Shaw et al., 1987). Different promoters can be fused to *luc* or *luxAB* for testing their strength and temporal and spatial expression (Maas et al., 1991, 1992). The advantages and disadvantages of using LUC or LUX genes as reporters in transgenic plants have been reviewed in detail (Koncz et al., 1990; Meighen, 1991).

The promoter of a reporter gene is very important for proper expression in transformed plants. The 35S promoter of cauliflower mosaic virus (CaMV) has been widely used for expression of reporter and other genes. It appears constitutively active in many plant species (Odell et al. 1985). A duplicated 35S promoter was constructed for enhanced expression of a fused gene (Kay et al., 1987). Strong promoters can be generated by inserting introns or 5'-nontranslated sequence of viruses between the 35S promoter and the gene. Insertion of maize *Adh-1* introns into the transcription unit increased the expression of reporter genes (Callis et al., 1987). The intron of maize *Shrunken-1* (*Sh-1*) gene enhanced reporter gene expression up to 1000-fold (Maas et al., 1991). The 5' nontranslated regions (5'UTRs) of tobacco mosaic virus enhances the translation of foreign genes in vivo and in vitro (Gallie et al., 1987). Similarly, the 5'UTRs of pea seedborne mosaic potyvirus (Nicolaisen et al., 1992) and potato virus X (Zelenina et al., 1992) all act as translational enhancers for increased expression of foreign genes. The untranslated leader sequence from the coat protein gene of alfalfa mosaic virus (AMV RNA4) was shown to enhance the translation efficiency of chimaeric mRNAs in in vitro translation systems (Jobling & Gehrke, 1987).

Agrobacterium-mediated transformation has been applied with many dicot plants. Recent studies have supported infection by *Agrobacterium* of some monocots. After infection and tumor formation in asparagus (*Asparagus officinalis*), nopaline, T-DNA and NPTII activity were detected in the transformed cells or in regenerated plants (Bytebier et al., 1987; Hernalsteens et al., 1984). The treatment of the monocot *Dioscoria bulbifera* (yam, Dioscoriaceae) with agrobacteria preincubated with wound substances from potato led to crown gall tumor formation (Schafer et al., 1987). These authors showed evidence of T-DNA integration into the yam genome and of nopaline production. Transformation by *A. tumefaciens* has also been demonstrated in narcissus (Amarylidaceae), *Chlorophytum capense* (Liliaceae), onion (Liliaceae), gladiolus (Iridaceae), maize (Gramineae), and wheat (Gramineae) (Creissen et al., 1990; Dommisse et al., 1990; Gould et al., 1991; Graves & Goldman, 1986, 1987; Hess et al., 1990; Hooykaas-Van Slogteren et al., 1984; Mooney et al., 1991; Raineri et al., 1990). In the Araceae, only calla and philodendron were reported to be susceptible to infection by *A. tumefaciens* strain B6 (De Cleene, 1985). Recently, Kuehnle and Sugii (1991b) reported tumor formation and nopaline production in *Anthurium andraeanum* when co-cultivated with *A. tumefaciens* in an induction medium containing AS. DNA amplification by PCR (polymerase chain reaction) and hybridization analyses indicated the presence of the nopaline synthase (NOS) gene of T-DNA in the plant tissue.

While most of the plant transformations by *Agrobacterium* were done by cocultivation with in vitro explants, some modified approaches using *Agrobacterium* were developed. *A. tumefaciens* carrying NPTII gene in the vector pGV471 and pGV472 was injected into immature *Brassica oleracea*

var. *botrytis* seeds. DNA analysis and NPTII enzyme activity assays demonstrated the integration of NPTII gene in plants (Eimert et al., 1992). Hess et al. (1990) applied *Agrobacterium* directly into the spikelets of wheat (*Triticum aestivum* L.) and 1%-2.6% of resulting wheat grains germinated on kanamycin-containing medium. Kanamycin- resistant seedlings were proved transformed by DNA analysis. Previously, the same group mixed wild type *A. tumefaciens* carrying pTiC58 plasmid with petunia pollen and pollinated the stigma. They reported tumor formation in seedling cotyledons of the progenies derived from this pollination (Hess & Dressler, 1989). Using GUS as a reporter, it was shown that foreign DNA integrated into pollen genome of petunia (Süssmuth et al., 1991).

2.3.2 Particle gun

The recent development of particle gun technology (microprojectile acceleration, or biolistic process) (Sanford et al., 1987) has made possible the transformation of previously recalcitrant crop plants. This technology bypasses the problem of host-specificity using *Agrobacterium* and has been successful with numerous dicot and monocot crops. Transient expression of reporter or marker genes has been reported in various tissues or cells after particle delivery. Stable transformation has been reported in monocots such as maize, and dicots such as soybean and papaya (Birch & Franks, 1991; Klein et al., 1992). Vasil et al. (1992) obtained fertile transgenic wheat with herbicide resistance by microprojectile bombardment of callus tissues. These authors felt that the key to the success is in regenerable embryogenic callus. GUS activity in herbicide resistant calli was very low. Only three out

of 82 resistant callus lines showed GUS activity by enzymatic staining. The authors speculated that weak expression of the GUS gene might be due to its being driven by the *Adh1* promoter.

Different designs of the particle gun have been reported. The original instrument uses the force from gun powder explosion to accelerate DNA coated tungsten microparticles to target cells via a macrocarrier (Sanford et al., 1987). The transformation efficiency has been improved by replacing the gun powder with helium gas and the macrocarrier with a plastic membrane (Sanford et al., 1991). Alternatively, the force accelerating the DNA carriers can be provided by an electric discharge mechanism (Christou, 1990). This approach has been successfully used in transforming immature zygotic embryos of rice (Christou et al., 1991) and soybean (McCabe et al., 1988).

Other simple and nonexpensive devices based on helium gas (Finer et al., 1992; Takeuchi et al., 1992) or compressed air (Iida et al., 1990; Morikawa et al., 1989; Oard et al., 1990) have been developed. Recently, Sautter et al. (1991) developed a micro-targeting accelerator for the acceleration and transfer of DNA-coated microprojectiles. The advantage of this device is that particles containing DNA can be dispersed in precise and restricted tissues, such as the meristem. The efficiency of stable transformation of tobacco is one plant per 103 cells that were exposed to the particles containing DNA (Sautter et al., 1992).

2.3.3 Electroporation, PEG- and liposome-mediated transformation of protoplasts

When the plant cell wall is removed by enzymatic digestion, the resulting protoplasts can be used for DNA uptake using chemicals such as polyethylene glycol (PEG) (Negrutiu et al., 1987). Protoplasts have been used for transient gene expression assay for the assessment of promoter and gene combinations. Electroporation has been used widely to introduce gene constructs into protoplasts and to assay for transient expression (Potrykus et al., 1985). The limitation of stable transformation depends on the regenerability of the protoplasts. Guerche et al. (1987) studied optimal conditions for electroporation of tobacco mesophyll protoplasts based on transient expression assay of chloramphenicol transacetylase activity (CAT) or scoring colonies expressing resistance to paromycin. The optimal voltage was 250-300 V/cm. Potassium chloride was the best electrolyte. The physiological state of the donor plants significantly affected the transformation ability of the protoplasts. Transient expression of luciferase (LUC) was studied in maize protoplasts isolated from embryogenic callus (Planckaert & Walbot, 1989). The maize *Adh1* intron inserted between 35S promoter and reporter genes (*cat, luc*) enhanced transient expression in electroporated protoplasts of breadwheat (Oard et al., 1989) and maize (Planckaert & Walbot, 1989).

Rathus and Birch (1992) obtained optimal conditions for electroporation of sugarcane protoplasts. Transient expression of the reporter genes for GUS and CAT was monitored after various electroporation conditions. Vasil et al. (1989) studied transient expression of the CAT reporter gene with

different promoters (*35S* vs maize *Sh1*) and with or without maize alcohol dehydrogenase (*Adh1*) or *Sh1* introns. The results indicated that 35S was better than *Sh1* promoter, and this promoter with the *Sh1* first intron gave highest level of transient expression.

Stable transformation has been obtained in rice after electroporation of protoplasts with pBI121 and pUC19-HPT (co-transformation) (Shimamoto et al., 1989; Tada et al., 1990). Transgenic soybean was obtained after electroporation of cotyledon protoplasts (Dhir et al., 1991). The transformation frequency was 23% based on hygromycin-resistant calli. Electroporation of tomato mesophyll protoplasts with vectors containing the kanamycin resistance gene resulted in stable transformation. The transformation efficiency was between 0.2% to 2% (Bellini et al., 1989).

Liposome-mediated gene transfer into plant protoplasts has been reported in maize cell lines (Antonelli & Stadler, 1990) and tobacco mesophyll protoplasts (Spörlein & Koop, 1991). In the latter, transformation was confirmed by germinating transformed, selfed seeds in kanamycin containing medium. Use of cationic liposomes (lipofectin) and electroporation was comparable to those achieved with PEG and/or electroporation. Liposomes were also used in lentil protoplasts transfection to study transient expression of reporter genes, such as *gus* and *cat* (Maccarrone et al., 1992).

Lyznik et al. (1989) compared the efficiency of PEG and electroporation methods to transform maize protoplasts. They found PEG is better than electroporation in terms of kanamycin-resistant colony recovery. Maas and Werr (1989) determined optimized conditions for PEG-mediated transformation of maize and rice protoplasts. DNA was precipitated by PEG

in the presence of divalent cations at optimal pH 6-6.5. Under these conditions, protoplasts uptake DNA efficiently.

Datta et al. (1990) were able to obtain fertile transgenic rice using the PEG method with a regenerable microspore-derived embryogenic cell suspension culture. Hygromycin was used for selection of transformed calli. Fertile transgenic rice plants were also obtained by other groups using PEG-mediated transformation (Hayashimoto et al., 1990; Peng et al., 1992). Lee et al. (1991) obtained transgenic rice expressing GUS activity after PEG-mediated transformation. Lazzeri et al. (1991) studied PEG-mediated DNA uptake into barley protoplasts. Under the G418 antibiotic selection, they obtained stably transformed calli. Karesch et al. (1991) obtained transgenic *Arabidopsis* plants by PEG-mediated DNA uptake into protoplasts. An average of 100 transformants were selected per 10^6 treated protoplasts in hygromycin containing medium. The efficiency of transformation is 0.1%.

2.3.4 Microinjection

Microinjection of DNA into protoplasts or cells to obtain transgenic plants has not received much attention, partly due to its tedious procedure and low frequency of cell survival. Toyoda et al. (1990) introduced GUS gene into barley coleoptile cells by microinjection. Maximum transient expression was detected 24 hours after injection. No stable transformation was reported in their study. Transgenic callus or transgenic plants have been reported in several studies (Neuhaus & Spangenberg, 1990).

Microinjection of DNA into proembryos and meristematic tissues may have more chance to obtain transformed plants (Potrykus, 1990).

Microinjection of DNA into microspore-derived proembryos has produced transgenic rape (Neuhaus et al., 1987). The drawback of this method is that many transgenic plants coud be chimeras of transformed and nontransformed tissues.

2.3.5 Macroinjection

Macroinjection means direct injection of DNA solution into plant tissue without use of a microscope. Croughan et al. (1989) injected a DNA solution into the stem of rice plants containing immature inflorescences. The DNA vectors contained kanamycin resistance marker and antibacterial genes, cecropin B or modified cecropin SB-37. They were able to recover kanamycin resistant plants by germinating seeds in the antibiotic medium. Seven out of 3922 seedlings (0.2 %) were transformed based on Southern blot analysis. De la Peña et al. (1987) obtained transgenic rye plants by injecting the DNA vector pGLVneo1103, carrying the kanamycin resistance gene, into young floral tillers. Two kanamycin resistant seedlings were recovered from 3023 seeds harvested from 98 plasmid- treated plants. Kanamycin activity and DNA analysis confirmed the stable integration of the NPTII gene.

2.3.6 Pollen tube pathway

This method is somewhat similar to the macroinjection method for rye transformation. Transgenic rice plants carrying the NPTII gene were obtained after application of a high concentration of plasmid DNA (50

mg/ml) onto a three-quarter-excised rice floret (Luo & Wu, 1989). Twenty percent of the rice seeds from treated florets contained NPTII gene however, this work has been not reproducible. Ohta (1986) reported that maize can be transformed by mixing pollen and DNA and applying it to the silks. Kameya et al. (1992) used tobacco flower to test the pollen tube pathway. The observation showed that the optimal time for transformation was right before or after fertilization (36 to 48 hours after pollination). Molecular evidence supported the integration of NPTII gene in tobacco.

2.3.7 Agroinfection and virus vectors

Agroinfection is defined as *Agrobacterium*-mediated virus infection (Grimsley et al., 1986). Wheat dwarf virus (WDV), maize streak virus (MSV), and cassava latent virus (CLV) all belong to the geminivirus group of plant viruses. Cloned virus DNAs were inserted into binary vectors and introduced into *Agrobacterium*. The *Agrobacterium* cells were directly inoculated onto their host plants or indicator plant tobacco. The host plants, wheat (Woolston et al., 1988) and maize (Grimsley et al., 1987), developed typical virus disease symptoms. The infectivity of modified CLV (containing CAT gene) was detected on tobacco (*Nicotiana benthamiana*) by assaying for chloramphenicol acetyltransferase activity (Ward et al., 1988).

Cloned cDNA of tobacco mosaic virus (TMV) was modified by removing the coat protein gene and replacing it with the CAT gene. When the in vitro transcripts from the coatless clone were inoculated on the local lesion tobacco plants, CAT activity was detected in the inoculated leaves. When transcripts of original infectious clone were inoculated to the tobacco, TMV-

specific local lesions were produced (Takamatsu et al., 1987). All these results imply that virus may be used as a plant expression vector to produce useful molecules.

2.3.8 Other methods of gene transfer

Silicon carbide fibers (average diameter 0.6 um, length 10-80 um) have been used for the delivery of DNA into maize (Black Mexican Sweet) and tobacco cells (Kaeppler et al., 1990) by vortexing. The average frequency of GUS expression in transient assay was 139.5 foci (equivalent to the blue spot on the cell layer) per sample. The same group later reported stable transformation of maize and tobacco cells using this approach (Kaeppler et al., 1992).

Another approach used is ultrasonication for DNA uptake by sugarbeet and tobacco protoplasts, sugarbeet and tobacco suspension cells (Joersbo & Brunsted, 1992). Intact leaf pieces of tobacco (4 x 8 mm) can also uptake DNA by sonication (Zhang et al., 1991).

Griesbach (1992) reported that *Calanthe* orchid leaves expressed GUS activity after exposure of the apical meristem of germinated protocorms to an electric field in the presence of pBI121 vector. Fifty-seven percent of the treated protocorms showed GUS-positive staining.

2.4 TISSUE CULTURE OF ANTHURIUM AND OTHER AROIDS

2.4.1 Callus culture

Regeneration of *Anthurium andraeanum* and *A. scherzerianum* has been obtained via a callus stage from cultured embryos and explants of leaf lamina, petiole, spadix (inflorescence stalk), and spathe (Finnie & van Staten, 1986; Geier, 1986, 1990; Pierik, 1975, 1976; Pierik et al., 1974, 1979). For callus induction in anthurium, usually auxin alone, or auxin and cytokinin are necessary in the culture medium (Finnie & van Staden, 1986; Kuehnle & Sugii, 1991a). The time required for callus formation in leaf explants, depending on the genotype, usually takes from 2 weeks to 3 months. Shoot initiation from the callus takes from 4 weeks to 6 months (Geier, 1990).

Pierik et al. (1974) used young parts of mature *Anthurium andraeanum* plants to induce callus on a modified Murashige and Skoog medium supplemented with the cytokinin N-benzyl-9-(2-tetrahydropyranyl)-adenine (PBA). The optimal temperature for callus growth in the dark is 25^{o}C. But the rate of callus growth varied considerably among different genotypes. Callus was induced to form adventitious shoots under the light. Kuehnie and Sugii (1991a) used leaves of several Hawaiian anthurium cultivars ('Kaumana', 'Kozohara', 'Marian Seefurth', 'Mauna Kea', 'Nitta', 'Ozaki', and 'Paradise Pink') to study suitable media for callus induction. Leaf explants produced callus after 2 to 3 months on a modified Pierik medium containing 0.36 uM 2,4-dichlorophenoxy-acetic acid (2,4-D) and 4.4 uM 6-benzyladenine (BA). Long-term cultures of callus of some anthurium

selections could form shoots spontaneously in the dark. They speculated some callus could be embryogenic. Their results also showed differences in callus formation among different genotypes.

Callus was produced in taro (*Colocasia esculenta*, an edible aroid) when the shoot tip explants were cultured on modified Nitsch medium with 2,4-D and BA (Malamug et al., 1992a). Shoot formation from callus was observed only in medium with 0.2, 0.4 or 0.6% agar. BA (1 mg/l) is required for shoot regeneration. Further multiplication of shoots was enhanced by adding the medium with BA and naphthalene acetic acid (NAA) at 1 mg/l each (Malamug et al., 1992b). Yam et al. (1990) induced callus formation by culturing axillary buds of taro (*Colocasia esculenta* var. *esculenta*) in a modified Murashige-Skoog (MS) medium containing taro extract and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). The induced callus was compact, slow-growing, and formed a few plantlets. When the axillary buds were cultured in medium containing 5 mg/l each of NAA and BA, they produced a fast growing, friable and morphogenetically active callus. Plantlets were generated after transferring the friable calli to 2,4,5-T containing medium. Another taro cultivar (*C. esculenta* cv. Akalomamale) was used for callus induction from axillary buds. NAA at 1 mg/l level along with taro corm extract was able to induce callus and plantlet formation. BA was not required for plantlet formation. Etiolated stems of several taro cultivars were obtained by culturing shoot apices in the dark on MS medium supplemented with 0.2 mg/l NAA (Murakami et al., 1992). Friable calli were obtained from culture of stem segments in MS medium containing 2 mg/l each of 2,4-D and 2-isopentenyladenine (2-iP). Protocorm-like structures developed on a medium containing 0.2 to 2 mg/l NAA and 2 mg/l BA or 2iP (Murakami et

al., 1992). Shoots were regenerated from both calli and protocorm-like structures.

2.4.2 Axillary bud multiplication

Anthurium can be micropropagated without an intermediate callus stage by use of axillary bud explants (Kunisaki, 1980). The best result for a shoot multiplication of *A. andreanum* was from modified MS medium with 0.2 mg/l BA. When BA was increased, callus growth increased but shoots were stunted (Kunisaki, 1980). Voyiatzi & Voyiatzis (1989) used lateral bud tip of *Dieffenbachia exotica* cv. Marianna for shoot multiplication study. 2-isopentenyl adenine (2iP) or kinetin are necessary for shoot multiplication. A similar approach for the propagation of taro was reported by Sabapathy and Nair (1992). They could induce shoot formation, axillary buds and protocorm-like bodies in shoot apex culture. The effective hormone combinations were 5.5 mg/l NAA and 0.2 mg/l kinetin or 1.85 mg/l NAA and 2 mg/l kinetin supplemented with polyamine, arginine or ornithine.

2.4.3 Seed culture

Seed-derived progenies of anthurium are heterogeneous in appearance (Pierik et al., 1974), therefore tissue culture is a preferred method for propagation. However, anthurium seeds have been used for callus induction and subsequent shoot formation. Pierik et al. (1974) used 'embryos' (the seed coat was removed, but the explant may still include endosperm) of *A. andraeanum* for induction of callus in the dark. Callus formation could be

improved by inclusion of the cytokinin PBA in the culture medium. When calli were transferred into light, most formed shoots. Rosario & Lapitan (1981) induced callus formation in seed culture of a *A. andraeanum* hybrid ('Duang Smorn' x 'Kaonaiwan') in MS medium containing 20% coconut water (CW). Callus was subcultured on the same medium with 10 mg/l indole butyric acid (IBA), 10 mg/l kinetin and 25% CW. Under this condition, plantlets were obtained after 7 months. The callus cultures were maintained in the dark. When regenerated etiolated shoots were transferred to light, plantlets turned green. When the embryo was excised and cultured without endosperm, embryos usually died or failed to produce callus. The formation of callus occurred at the embryo end of the seed (Rosario & Lapitan, 1981).

2.4.4 Spadix culture

Geier (1982, 1990) reported that spadix-derived callus of *A. scherzerianum* regenerated plantlets, and possibily produced somatic embryos. Some of the putative somatic embryos developed into early-phase bipolar seedling-like structures, but in most cases, they developed into clumps with multiple shoots and roots.

2.4.5 Liquid culture

The growth of callus tissue from *A. andraeanum* genotype A42-3 in liquid media was studied by Pierik (1975). The multiplication rate for the callus in term of fresh weight increase varied considerably among different

genotypes. Different genotypes had different response. In this study, callus growth in liquid media seemed unaffected by the presence of the cytokinin PBA. Pierik (1976) further studied 38 genotypes of anthurium in liquid culture. The speed of callus formation and the quantity of callus per explant is strongly dependent on genotype. 31 out of 38 genotypes could form callus and subsequent subcultures. In this study, PBA was included in liquid medium for callus subculture and shoot regeneration.

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CHAPTER 3

TISSUE CULTURE AND REGENERATION OF ANTHURIUM

3.1 INTRODUCTION

Methods for reliable tissue culture and plant regeneration from different tissues or organs are required for genetic transformation of anthurium. The first report of callus induction and plant regeneration from zygotic embryo and seedling tissues, was described by Pierik et al. (1974). Since then, regeneration of anthurium plantlets through the callus pathway was reported for various tissues, including leaf lamina, petiole, spadix, spathe, and flower stem (reviewed by Geier, 1990; see also this Literature Review).

In a comparison of *A. andraeanum* Hort. leaf lamina, petiole, spathe, spadix and root explants, Finnie and van Staden (1980) found root tissues were capable of callus production but required longer periods of culture than either lamina or petiole tissues; the latter were reported to be the most responsive explants. Using several Hawaiian cultivars, Kuehnle and Sugii (1991a) found lamina explants more responsive than petioles for callus formation. Putative somatic embryos were observed in some highly regenerative calli in this study. Somatic embryogenesis was also observed in cultures of *A. scherzerianum* spadices, although there was no indication of regeneration (Geier, 1982).

An alternative to callus or somatic embryogenesis for plant regeneration from anthurium explants is direct shoot formation from axillary buds

(Kunisaki, 1980). Although use of this explant may reduce the risk of generating somaclonal variation, the number of buds available for any one genetic transformation experiment is relatively low.

This chapter describes methods for culture of anthurium etiolated internode, lamina and root explants and subsequent plantlet regeneration via shoot formation or somatic embryogenesis.

3.2 MATERIALS AND METHODS

3.2.1 Plant materials

University of Hawaii advanced selections UH965 and UH1060 were used in all the experiments. UH965 (Fig. 3-1A) is recently released as cultivar 'Rudolph' (Kamemoto et al., 1992). Its parentage is shown in Fig. 3-1B. UH1060 (Fig. 3-2A) is a species hybrid between *A. andraeanum* and other species (H. Kamemoto, personal communication). Its parentage is shown in Fig. 3-2B. Explants were obtained from established plants grown in vitro in Magenta GA-7 boxes (Sigma Chem. Co., St. Louis, Missouri) containing a modified Murashige and Skoog (Murashige & Skoog, 1962) medium (MS) with 0.2 mg/l 6-benzyl adenine (BA, Sigma Chem. Co.) (Kunisaki, 1980; Kuehnle and Sugii, 1991a); this medium is designated H1 (Table 3-1).

Etiolated internodes of UH1060 were obtained from shoots formed from node cultures in H1 medium in the dark at 23^{o}-25^{o}C. In vitro lamina explants were obtained from plantlets grown on H1 medium in GA-7 boxes.

Fig. 3-1A. The inflorescence of a UH965 ('Rudolph') plant.

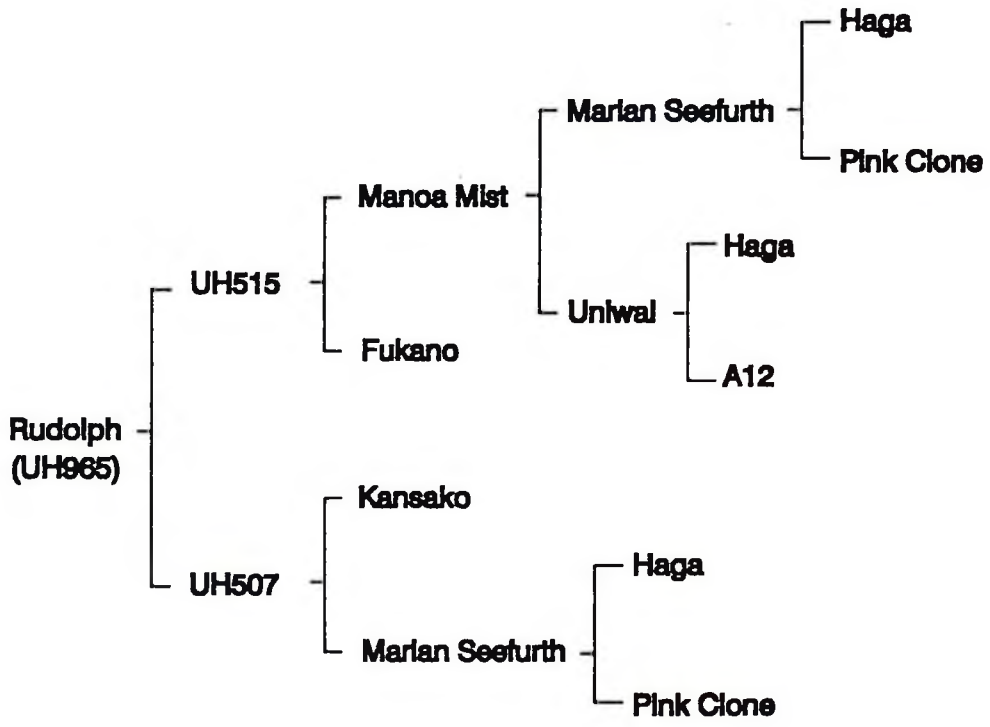

Fig. 3-1B. Pedigree of UH965 ('Rudolph').

Fig. 3-2A. The inflorescence of a UH1060 plant.

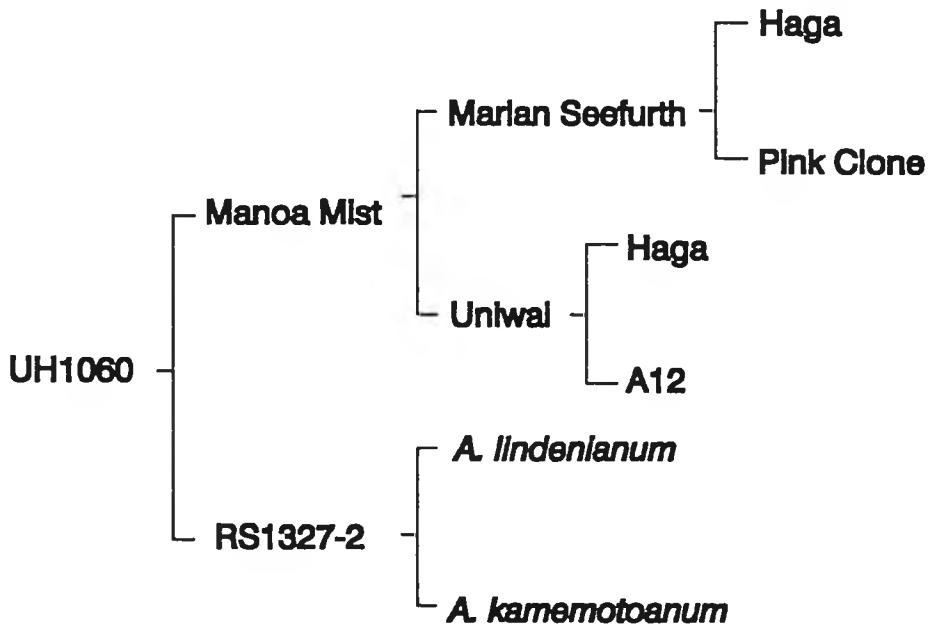

Fig. 3-2B. Pedigree of UH1060.

3.2.2 Media

A basal medium of half-strength MS macronutrients, full strength MS micronutrients, 0.4 mg/l thiamine·HCl, 0.5 mg/l each of nicotinic acid and pyridoxine·HCl, and 100 mg/l myo-inositol was used (Table 3-1). Three media were tested for callus formation and somatic embryogenesis: C, F, and D with variable amounts of 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma Chem. Co., St. Louis, Missouri) and cytokinins (BA, 2iP, or kinetin). Medium D is the same as basal medium reported by Kuehnie and Sugii (1991a) except the auxin 2,4-D was modified. Media 1/2MSO and H1 were used for somatic embryo conversion. H1 medium (Kunisaki, 1980) was used for etiolated shoot culture and for root segment culture. The amount of sugar was either 20 or 30 g/l sucrose and 0 or 10 g/l glucose. The amount of NaFeEDTA (Sigma Chem. Co.) was either 25.7 or 36.7 mg/l, as shown in Table 3-1. The media were solidified with 7 or 7.5 g/l of Difco-Bacto agar (Difco Laboratories, Detroit, Michigan) or 2 g/l Gelrite (Scott Laboratories, Carson, California).

3.2.3 Culture of internodes and laminae

Callus formation in internodes. A screening of media with combinations of 2,4-D and kinetin (Sigma Chem. Co.) was initiated during January, 1990. The purpose was to find an effective growth regulator combination for callus formation from etiolated internodes. The basal medium was D medium (Table 3-1). Media were solidified with 2 g/l Gelrite (Scott Laboratories).

Table 3-1. Composition of media used in tissue culture of anthurium

Components	Medium and composition				
	1/2MSO	D[a]	C	F	H1
Macronutrients	1/2x MS	1/2x MS	1/2x MS	1/2x MS	1/2x MS
Micronutrients	MS	MS	MS	MS	MS
NaFeEDTA	25.7 mg/l	25.7 mg/l	25.7 mg/l	25.7 mg/l	36.7 mg/l
Vitamins	As MS[b]	As MS[b]	As MS[b]	As MS[b]	As MS[b]
Sucrose	30 g/l	20 g/l	20 g/l	20 g/l	20 g/l
Glucose	0	10 g/l	10 g/l	10 g/l	0
Myo-inositol	100 mg/l	100 mg/l	100 mg/l	100 mg/l	100 mg/l
2,4-D	--	--	1.5 mg/l	3.0 mg/l	--
BA	--	--	--	--	0.2 mg/l
Kinetin	--	--	0.5 mg/l	0.5 mg/l	--

[a]From Kuehnle & Sugii (1991a), with altered growth regulator levels
[b]With 0.4 mg thiamine·HCl/l

Factorial combinations of 2,4-D (1.5 mg/l and 2 mg/l) and kinetin (0, 0.5, 1, 2, 4, and 8 mg/l) were added to the basal medium. After autoclaving, media were dispensed in a 24-well plate (Falcon 3047, Becton Dickinson & Co., Lincoln Park, New Jersey) with 2 ml per well. Etiolated internode explants about 0.5 cm long were placed in each well, 3 pieces per well. Callus formation on internodes was observed, but not quantified, after a 6-week period of culture in the dark at 23°-25°C.

Callus formation and somatic embryogenesis in lamina culture. Based on the result of the above experiment, intact laminae were used to test for callus formation and somatic embryogenesis. In vitro lamina culture was initiated during March and July, 1990. In vitro-grown leaf laminae of UH1060 were severed from petioles. Laminae were placed in 10-cm plastic Petri dishes containing basal medium D (Table 3-1) and 3 mg/l of 2,4-D and 0.5 mg/l of kinetin (designated as F medium, Table 3-1). Each plate contained 10 or 11 laminae and was replicated eight times. The plates were incubated at 23°-25°C in the dark. After 4 months, laminae of one plate were transferred to freshly prepared F medium. All others were transferred to C medium, which is the same as F medium except the 2,4-D level decreased to 1.5 mg/l (Table 3-1). Following an additional 10 days (a total of 130 days) of culture, the number of explants forming callus and somatic embryos (including primary and secondary embryos) was scored.

To observe conversion and plant regeneration of lamina-derived somatic embryos in the light, four somatic embryos were removed carefully and transferred to a hormone-free medium containing 3% sucrose (designated 1/2MSO, Table 3-1) solidified with 7 g/l Difco Bacto-agar (Difco

Laboratories, Detroit, Michigan). They were cultured in low light (4 $\mu Em^{-2}sec^{-1}$, 16 hours light/8 hours dark) at 25°C.

Observations of somatic embryos were also made using scanning electron microscopy (SEM). The procedure for SEM was basically according to Vasil and Vasil (1984) with some modifications. One piece of embryogenic callus from lamina culture was fixed in glass vials containing 2.5% (v/v) glutaraldehyde in 0.1 M sodium-cacodylate buffer (pH 7.4) for 1 hour at 25°C. The tissues were washed with three changes of cacodylate buffer for 30 minutes (10 minutes each). The samples were post-fixed in 1% (w/v) osmium tetroxide in cacodylate buffer for 2 hours at 4°C. The samples were washed with two changes in cacodylate buffer for 20 minutes. The tissues were then dehydrated for 1 hour each in 25, 50 and 75% ethanol with gentle agitation every 15 minutes. At this point, the samples were stored at 4°C overnight. The next day, they were transferred into 100% ethanol, with two additional changes before critical-point drying. The samples were critical-point dried in an Autosamdri-810 instrument (Tousimis Scientific Instrument, Rockville, Maryland). The samples were coated with gold/paladium particles using a Hummer II Sputter Coater (Anatech Ltd., Alexandria, Virginia) and viewed with a Hitachi 800 scanning electron microscope under 10 kilovolts (kV).

3.2.4 Effect of calcium concentration on somatic embryogenesis

The calcium amount in C medium (1.5 mM) was adjusted with a calcium chloride solution so that the final concentrations were: 1.5, 2.0, 2.5, 3.0, 6.0, and 8.0 mM. Laminae from in vitro grown plantlets of UH1060 were

examined for somatic embryogenesis after 95 days of culture in these six media. The media were dispensed into 10-cm diameter plastic Petri plates, two plates per treatment. Laminae were placed on top of the media, 15 laminae per plate. The plates were incubated in the dark at 23^{o}-25^{o}C.

3.2.5 Effect of cytokinins on organogenesis and somatic embryogenesis

Laminae derived from in vitro plantlets of UH965 and UH1060 were used in this experiment. The basal medium D (Table 3-1) was supplemented with different cytokinins. The cytokinin levels used were as follows: kinetin--0.5, 1, 2 mg/l (designated CK1, CK2, and CK3); N^{6}-[2-isopentenyl] adenosine (2iP, Sigma Chem. Co.)--1, 1.5, 3 mg/l (designated CK4, CK5, and CK6); BA and 2iP combined--0.2 and 3 mg/l respectively (designated CK7); and BA--0.2 mg/l (designated CK8). The occurrence of shoots or somatic embryos were recorded after 3 months' culture in the dark at 23^{o}-25^{o}C.

3.2.6 Effect of 2iP and 2,4-D on the response of UH965 lamina culture

Factorial combinations of growth regulators 2iP (1.5 and 3.0 mg/l) and 2,4-D (0, 0.5 and 3.0 mg/l) were conducted to study the response of excised UH965 laminae. The basal medium was the same as D medium (Table 3-1). The media were solidified with 1.8 g/l Gelrite and dispensed in 10-cm plastic Petri plates (20 ml/plate). Ten or 11 laminae excised from in vitro-grown plantlets were placed on top of media. The explants were incubated in the

dark at 23°-25°C for 83 days. The number of explants forming roots or embryogenic callus was recorded.

3.2.7 Establishment of etiolated shoots from lamina culture

Leaves from in vitro grown anthurium plantlets were cultured on H1 medium solidified with Difco Bacto-agar (6 or 7 g/l) or Gelrite (1.76 g/l) in 10-cm plastic Petri dishes in the dark. After 2-3 months, regenerated shoots attached to the original laminae were transferred to Magenta GA-7 boxes containing H1 medium solidified with Bacto-agar (7 g/l) and further cultured in the dark for at least 4 months (Fig. 3-13). It may be noted that the nodes from etiolated shoots can continue to be cultured in the same medium after excising internodes for cocultivation experiments.

3.2.8 Root culture

In vitro grown roots were cut into about 1-cm long segments and cultured in 10-cm diameter plastic petri dishes containing H1 medium (Table 3-1) solidified with 1.76 g/l Gelrite. Each plate contained 25 root explants. One of the two plates was incubated under low light in a tissue culture room (4 $\mu E\ m^{-2}s^{-1}$, 16 hours light/8 hours dark cycle) at 25°C. The other plate was incubated in the dark at the same temperature. Callus and shoot formation were scored after 2 and 3 months, respectively.

3.3 RESULTS

3.3.1 Callus formation and somatic embryogenesis

Etiolated internode explants of UH1060 formed either callus or callus with roots (Fig. 3-3) when cultured for 41 days in various combinations of 2,4-D and kinetin. The most extensive callus growth was observed in combinations of 1.5 mg/l or 2 mg/l 2,4-D with either 0.5 mg/l or 1 mg/l kinetin. More callus growth appeared to occur on the medium with 1.5 mg/l 2,4-D and 0.5 mg/l kinetin (medium C) rather than on the medium with 2 mg/l 2,4-D and 0.5 mg/l kinetin.

Based on the observations using internodes, medium C was chosen to test for callus induction using intact lamina. However, due to an error during medium preparation, a second medium (F) of the same composition as medium C except with double the 2,4-D concentratiom (3 mg/l) was used. After 130 days, 36% of the lamina explants cultured on F medium alone formed embryogenic callus, while 52% of the explants cultured on F medium, then C medium, formed embryogenic callus (Table 3-2).

In all explants, the initial callus after 1 to 2 months in culture was translucent when examined with a dissecting microscope. Somatic embryos appeared later from these translucent calli (Fig. 3-4). Other embryos seemed to form directly from the cut edges of laminae. The morphology of these somatic embryos looked like dicot embryos, since they all had radicle-like

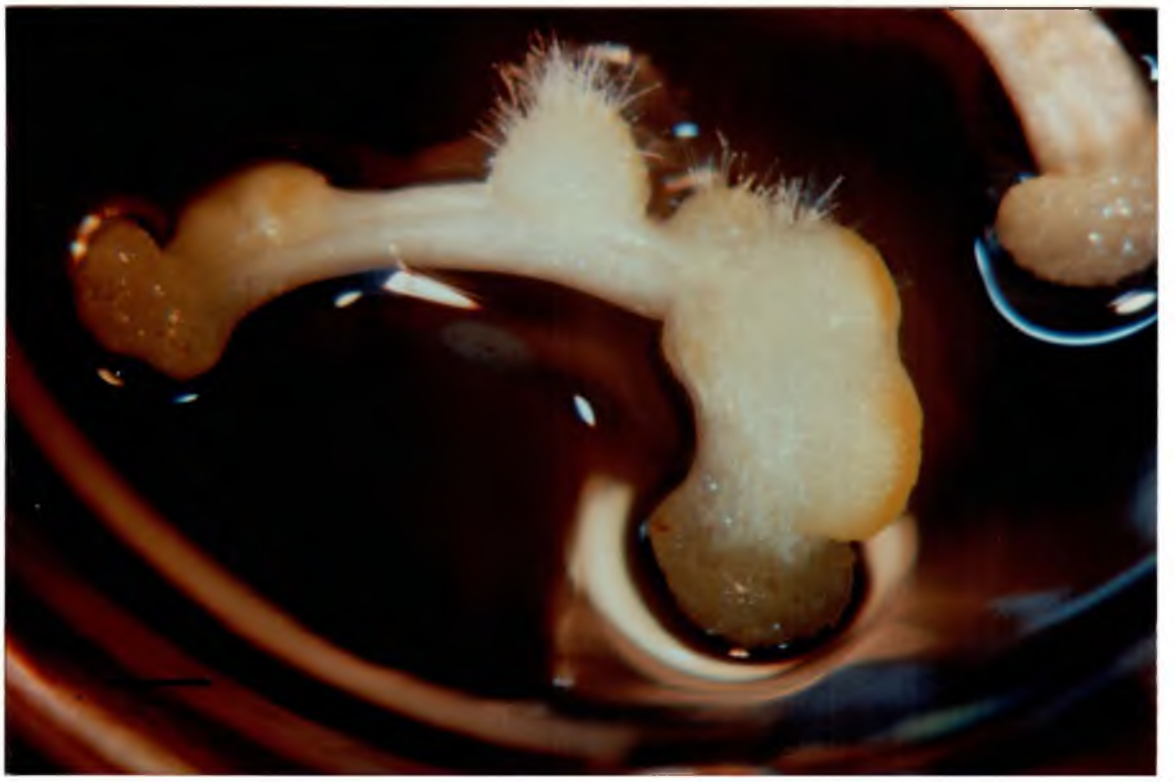

Fig. 3-3. Callus and root formation from internode culture of UH1060 on C medium. (Bar = 1 mm)

Table 3-2. Callus formation and somatic embryogenesis in UH1060 lamina culture

Treatment	Total No. explants	No. forming callus (%)		No. with SE[a] (%)	
F, then C medium	71	37	(52)	18	(25)
F medium only	11	4	(36)	0	(0)

[a]SE, somatic embryo, including primary and secondary somatic embryos

Fig. 3-4. Occurrence of somatic embryo from translucent callus of a UH1060 lamina culture on C medium. (Bar = 1 mm)

structures, and cotyledons (Fig. 3-5). Some embryos seemed to possess multiple or aberrant cotyledons (Fig. 3-5).

In about 3 to 4 months, secondary embryos were observed on the surface of the primary embryos (Fig. 3-6). These secondary embryos were discrete globular structures and occurred frequently on the surfaces of the primary embryos. When the embryogenic mass (including primary embryo and secondary embryos) was examined under the scanning electron microscope, the globular embryos were seen to be attached to the primary embryo without noticeable suspensors (Fig. 3-7). Some embryos possessed a notch, or invagination region (Fig. 3-7, arrow), from which it appeared that new shoots emerge. When the secondary embryo converts to a plantlet (Fig. 3-8), some secondary embryos converted directly at the place of their occurrence, but only converted into a shoot without root formation. Most of the primary embryos formed roots and shoots after long-term culture (4-6 months) on F and C medium.

Four somatic embryos were removed from the callused laminae and cultured on growth regulator free medium (1/2MSO). These embryos developed normally in 1/2MSO medium, but growth was slow. After about 3 months, a bipolar structure with cotyledons and radicle was clearly discernible (Fig. 3-9). Two embryos were then cultured on H1 medium in the light, and developed multiple green shoots and a large mass of green callus later. When other embryos were transferred to liquid C medium, they swelled and became callus-like in appearance.

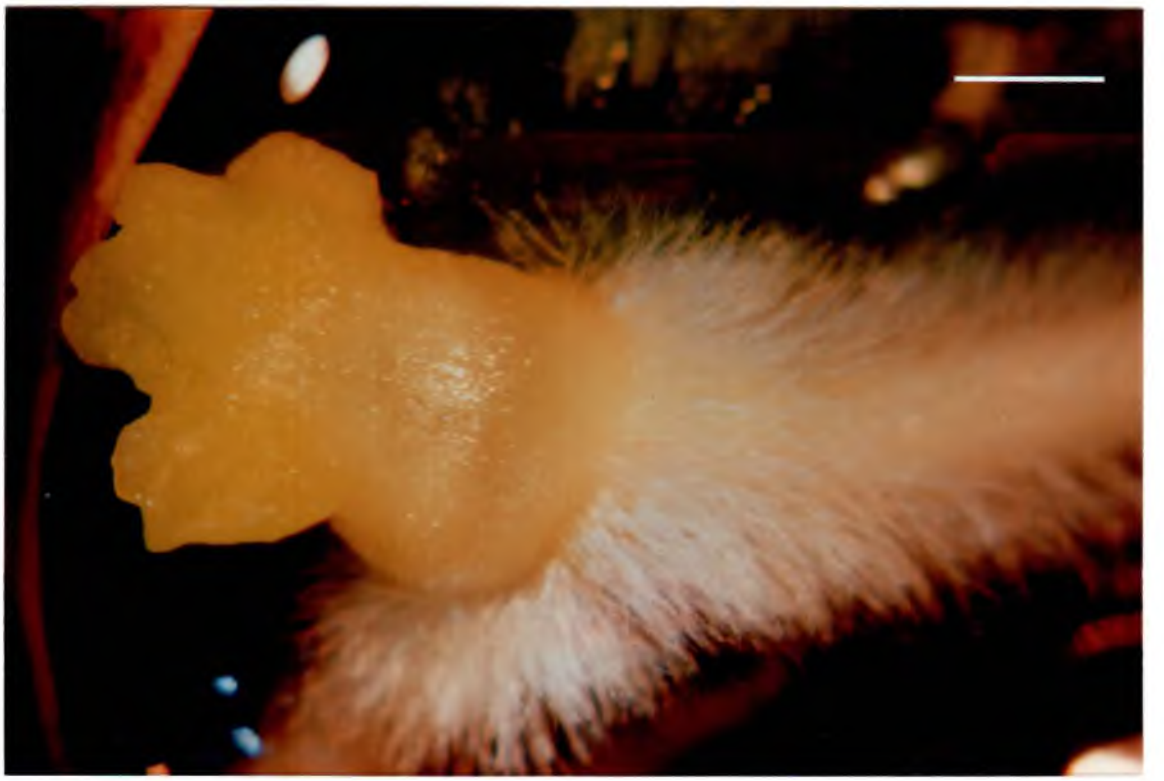

Fig. 3-5. Typical primary somatic embryo derived from lamina culture of UH1060 on C medium. Note the multiple cotyledon-like structure and the root. (Bar = 1 mm)

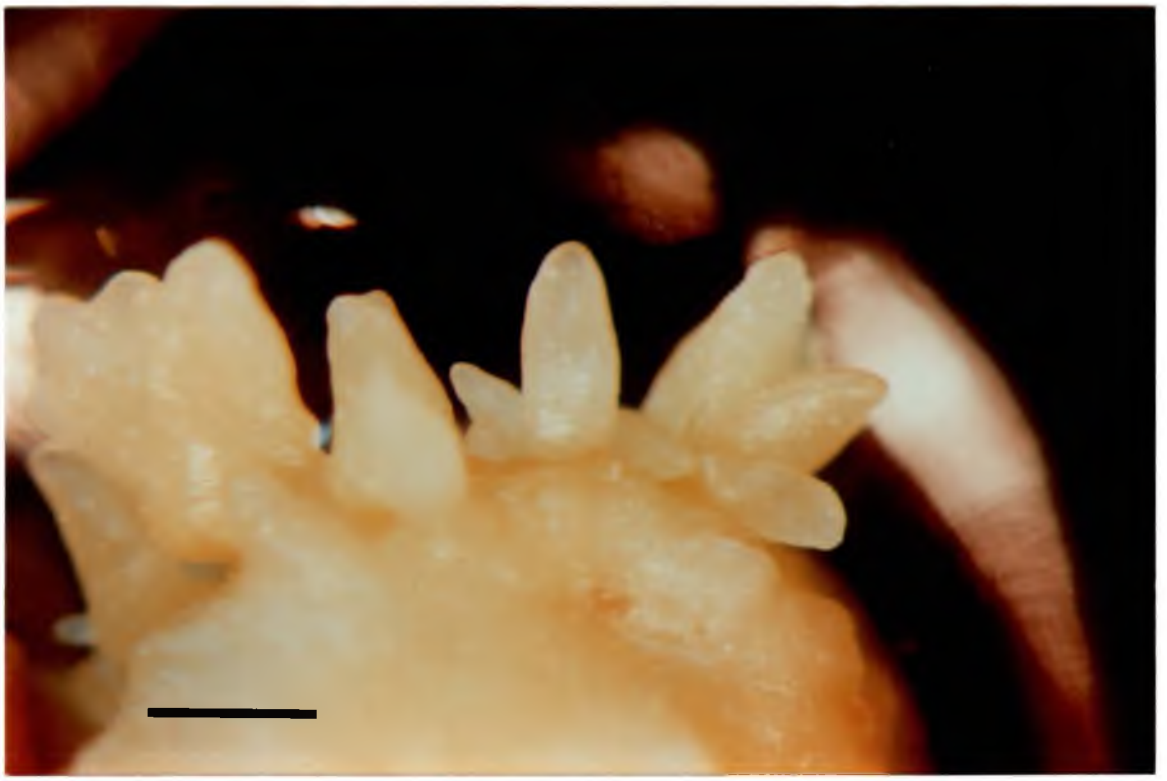

Fig. 3-6. Secondary somatic embryos derived from a primary embryo in lamina culture of UH1060 on C medium. (Bar = 1 mm)

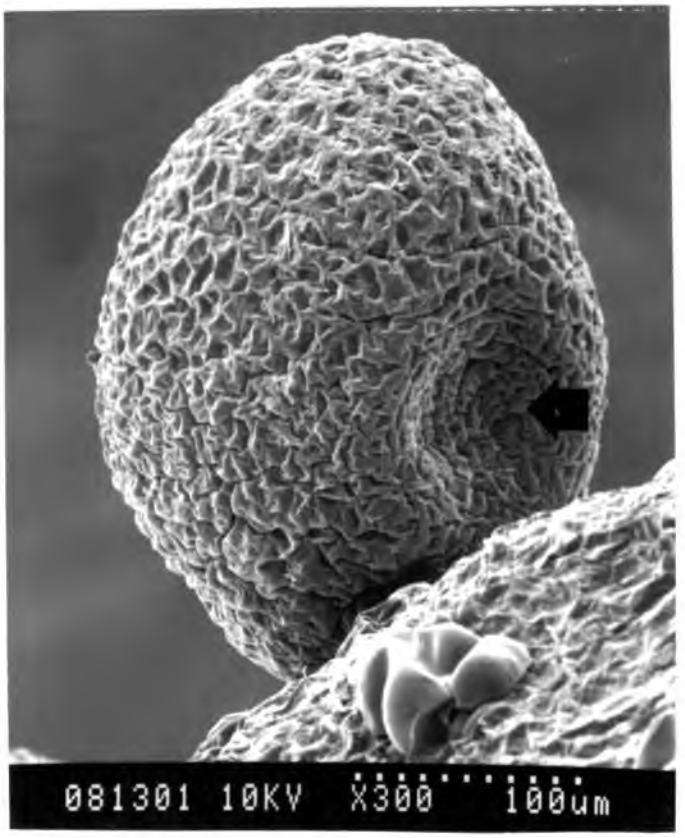

Fig. 3-7. Scanning electron micrograph of a globular secondary embryo with a notch region (arrow). Note the embryo attached to a primary embryo without suspensor.

Fig. 3-8. A new shoot emerges from the notch region of the secondary somatic embryo while still attaching to the primary embryo. (Bar = 1 mm)

Fig. 3-9. A converted somatic embryo grown on hormone-free 1/2MSO medium. (Bar = 1 mm)

3.3.2 Effect of calcium concentration on somatic embryogenesis

On C medium, 37% of the intact lamina explants of UH1060 produced somatic embryos at a Ca^{2+} concentration of 1.5 mM (Table 3-3). When the calcium concentration was increased, the percentage of laminae with somatic embryos increased to 53% at the 2.0 mM level, or to 43% at both the 2.5 and 3.0 mM levels. A calcium concentration higher than 3.0 mM prevented both callus and somatic embryo formation (Table 3-3). At 6.0 and 8.0 mM levels, the cut surface of the lamina base either turned brown or showed no response.

3.3.3 Cytokinins can induce shoots and somatic embryos on lamina cultures

An initial attempt at using cytokinin for shoot regeneration from intact lamina cultures was based on the observation by Kuehnle et al. (1992) that kinetin at 0.5 mg/l could induce shoot formation. Therefore, different cytokinins, including kinetin, BA and 2iP, were tested to induce shoot formation; this step could then be used to obtain regenerants following cocultivation of laminae with *Agrobacterium*.

The response of UH965 lamina culture is shown in Figure 3-10. In UH965, 0.5 mg/l kinetin induced 100% shoot formation from lamina culture. When kinetin was increased, shoot formation was reduced to 40% at 1 mg/l level, and callus was observed. Two mg/l of kinetin was inhibitory to both shoot and callus formation.

Table 3-3. Effect of calcium concentration on callus and somatic embryo formation on UH1060 intact lamina culture

Ca^{2+} concentration (mM)[a]	No. laminae cultured	No. forming callus	No. forming somatic embryos (%)
1.5	30	30	11 (37)
2.0	30	30	16 (53)
2.5	30	30	13 (43)
3.0	30	30	13 (43)
6.0	30	0	0 (0)
8.0	30	0	0 (0)

[a]In C medium containing basal salts and growth regulators as described in Table 3-1

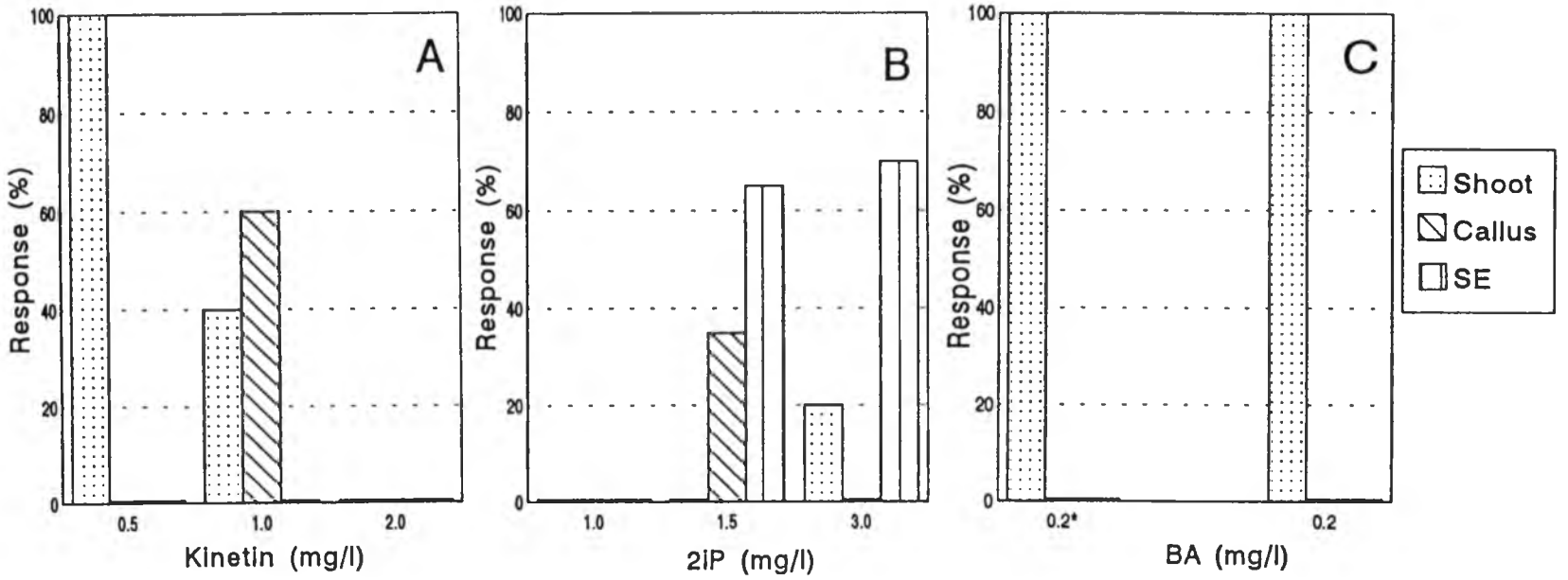

Fig. 3-10. Response of UH965 lamina culture to different cytokinins. (A), Kinetin; (B), 2-isopentenyl adenosine (2iP); (C), BA; *, with additional 2iP (3 mg/l); SE, somatic embryos.

The cytokinin 2iP at the 1 mg/l level had no effect on lamina culture. When 2iP was increased to 1.5 mg/l, both callus and somatic embryo formation were observed after 3 months, but no shoots formed. When 2iP was increased to 3 mg/l, the pattern was changed to shoot formation (20%) and somatic embryogenesis (70%). The occurrence of somatic embryos in UH965 on 2iP media took about 3 months. A typical somatic embryo with a suspensor emerging from the cut end of a lamina explant, is shown in Fig. 3-11. When 3 mg/l of 2iP was supplemented with 0.2 mg/l BA, all of the explants (100%) formed shoots, as did treatments with 0.2 mg/l BA alone (Fig. 3-10C) or with 0.5 mg/l kinetin alone. However, no somatic embryos formed.

The response of UH1060 lamina culture is shown in Figure 3-12. Like UH965, 0.5 mg/l kinetin induced 100% shoot formation in UH1060. When kinetin was increased to 1 mg/l, most of the explants produced callus (90%), while only a few (5%) produced shoots. Kinetin at 2 mg/l was again inhibitory to the shoot formation of UH1060. The cytokinin 2iP at 1, 1.5 and 3 mg/l levels had no effect on regeneration of UH1060. When 2iP (3 mg/l) was combined with 0.2 mg/l BA, 100% of the explants produced shoots. When BA (0.2 mg/l) was used alone, in addition to shoot formation (100%), some explants (10%) also produced somatic embryos.

3.3.4 Response of UH965 lamina cultures to 2iP and 2,4-D

Since somatic embryogenesis by UH965 lamina cultures was observed on media containing 1.5 mg/l and 3.0 mg/l 2iP, an experiment was conducted to see whether cytokinin (2iP) and auxin (2,4-D) together could induce

Fig. 3-11. A globular somatic embryo of UH965 occurred in the cut surface of lamina base cultured on medium with 3 mg/l 2iP. Note the suspensor (arrow). (Bar = 1 mm)

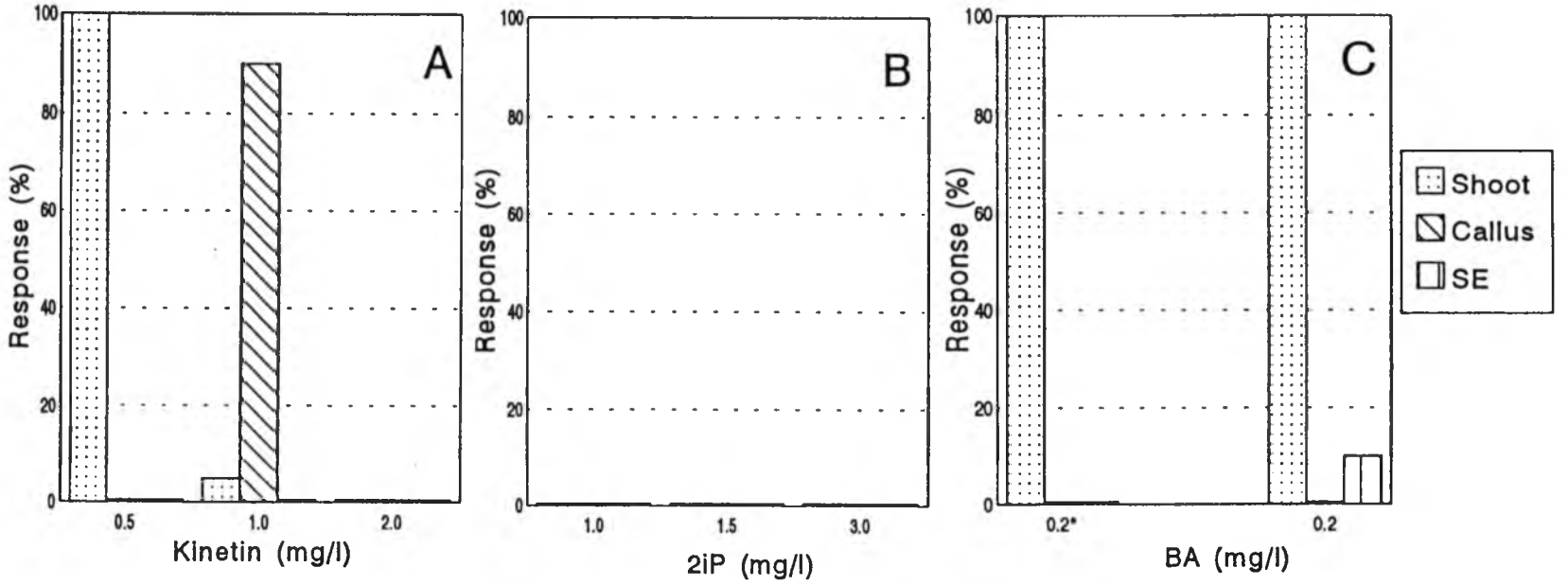

Fig. 3-12. Response of UH1060 lamina culture to different cytokinins. (A), Kinetin; (B), 2-isopentenyl adenosine (2iP); (C), BA; *, with additional 2iP (3 mg/l); SE, somatic embryos.

somatic embryogenesis as seen in UH1060 lamina culture on C medium (Section 3.3.1). As expected, 1.5 mg/l 2iP did not induce any embryogenic callus or roots (Table 3-4). When 1.5 mg/l 2iP was combined with 0.5 mg/l of 2,4-D, 15% of the explants formed embryogenic callus, and every explant also produced very short roots and a lot of root hairs. Further increasing the amount of 2,4-D only resulted in root formation.

When 3.0 mg/l 2iP was used alone, there was no root formation, and 65% of the explants formed embryogenic callus. When 0.5 mg/l of 2,4-D was added to the medium, 20% of the explants formed embryogenic callus. Increasing 2,4-D concentration decreased the percentage of somatic embryogenesis. No embryogenic callus was observed in the medium containing 3.0 mg/l each of 2iP and 2,4-D. Root formation was observed in the combinations with 3.0 mg/l 2iP and 0.5 mg/l to 3.0 mg/l 2,4-D (Table 3-4). In this experiment, media suitable for embryogenic callus formation of UH965 contained 2iP in concentrations of 1.5 to 3 mg/l and 2,4-D in concentrations between 0 and 1.5 mg/l.

3.3.5 Formation of multiple etiolated shoots from laminae

Cultured laminae formed multiple etiolated shoots after 4 months' culture on H1 medium in the dark. In UH1060, the number of etiolated shoots ranged from 10 to 20 per lamina. In UH965, short multiple shoots (about 0.5 cm long) formed in the responsive laminae. However, fewer than five shoots developed beyond 1 cm. An example of etiolated shoots from lamina culture of UH965 is shown in Fig. 3-13.

Table 3-4. Effect of 2iP and 2,4-D on root and somatic embryo formation on UH965 lamina culture

Regulators (mg/l)		No. laminae cultured	No. forming roots	No. forming embryogenic callus (%)	
2iP	2,4-D				
1.5	0	20	0	0	(0)
1.5	0.5	20	20	3	(15)
1.5	1.5	21	13	0	(0)
1.5	3.0	20	8	0	(0)
3.0	0	20	0	13	(65)
3.0	0.5	20	18	4	(20)
3.0	1.5	20	18	2	(10)
3.0	3.0	20	8	0	(0)

[a]Including somatic embryos

Fig. 3-13. Etiolated shoots of UH965 regenerated from lamina culture on H1 medium. (Bar = 10 mm)

Fig. 3-14. Shoot derived from a fragmented root of UH965 cultured on H1 medium. (Bar = 1 mm)

Table 3-5. Response of anthurium UH1060 root explants 3 months after culture in light or dark

Treatment[a]	No. explants cultured	No. forming callus	No. forming shoot[b]	% shoot regeneration
Dark	25	3	0	0
Light	25	20	18	72

[a]On H1 medium, except 0.18% Gelrite was used as solidifying agent
[b]Scored 3 months after culture

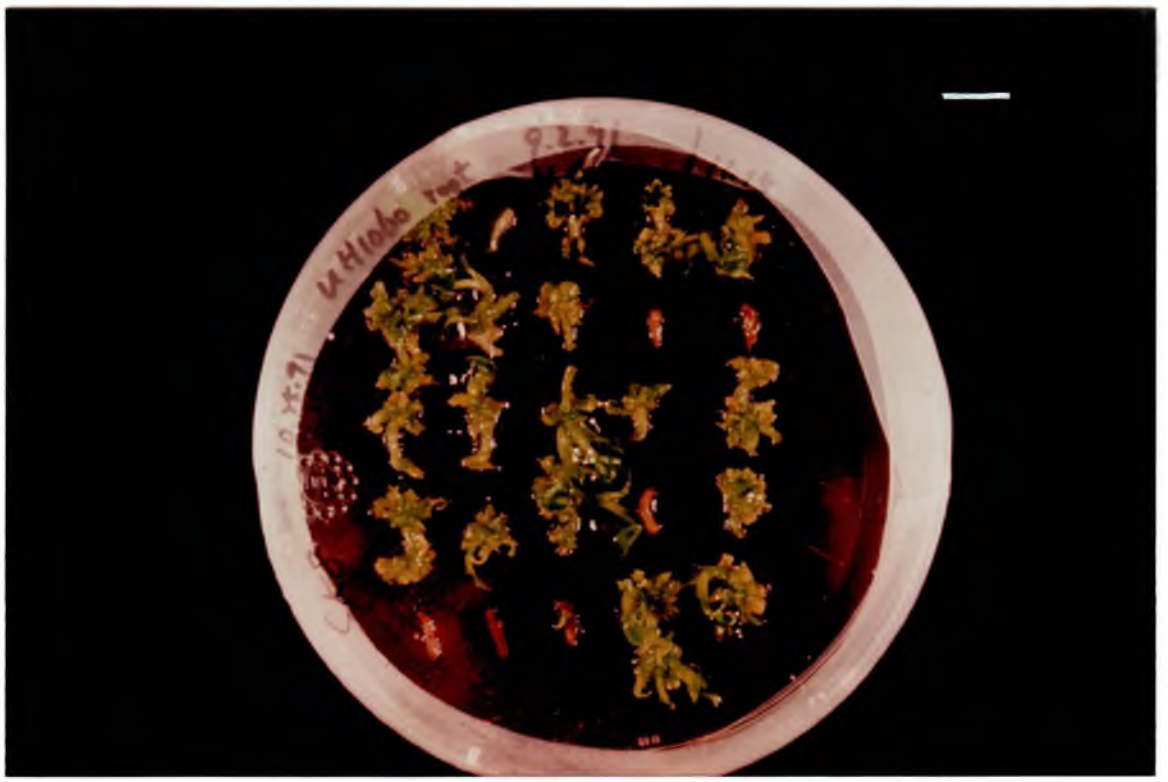

Fig. 3-15. Shoot regeneration from root segment culture of UH1060 on H1 medium. (Bar = 10 mm)

Fig. 3-16. Greenhouse-grown plants from UH1060 root segment culture on H1 medium. (Bar = 40 mm)

3.3.6 Root culture

Shoot formation from a root segment of UH965 was noted on one occasion while subculturing plantlets on fresh H1 medium. The shoot originating at one terminus of a root segment (Fig. 3-14) was observed. Subsequently, an experiment was initiated for root culture of UH1060, the only genotype available then. Under dark conditions, only three out of 25 explants formed small calli after 2 months, while 20 out of 25 explants formed callus under the light in the same period (Table 3-5). Most explants producing calli regenerated shoots in the light after 3 months of culture (Fig. 3-15). In some cases, only one end of the root segment produced callus. Some had callus on both ends. The regenerated shoots with roots were transferred after about 4 months to 5-inch pots containing tree-fern fiber. These plants were grown in the greenhouse for future evaluation of any variation. One year after initiation of the experiment, all the transplanted plants looked normal (Fig. 3-16).

3.4 DISCUSSION

Somatic embryogenesis occurred using UH965 and UH1060 in vitro-derived intact laminae using either cytokinin (kinetin) and auxin (2,4-D) together or cytokinin (2iP and BA) alone in culture media. Some secondary somatic embryos can germinate directly from the attachment site on the primary embryo. Some primary and secondary somatic embryos can be detached and cultured to form rooted plants with multiple shoots. The response of UH965 and UH1060 laminae by 2iP differed in terms of somatic

embryogenesis. Based on the occurrence of somatic embryogenesis in UH1060 lamina culture, further tests with different ranges of kinetin and 2,4-D combinations were performed with several anthurium cultivars (Kuehnle et al., 1992). Similar results (somatic embryogenesis) were obtained in media containing 2,4-D and kinetin. This confirmed that somatic embryos of anthurium could be obtained from lamina culture.

Kuehnle and Sugii (1991a) studied callus induction and plantlet regeneration in several Hawaiian anthurium cultivars. Lamina and petiole explants formed callus on most media tested. In general, leaves were more responsive than petioles in callus induction. A modified Pierik (1976) medium (Pmod) was recommended for callus induction. All long-term (12-13 months) cultures regenerated plantlets. Putative somatic embryos were observed in some calli. Explants from the spadix of *A. scherzerianum* were induced to produce callus and subsequent plantlet regeneration (Geier, 1982). Somatic embryogenesis was reported previously in spadix culture of *A. scherzerianum* (Geier, 1982), but no conversion of these embryos into plants was observed.

In *Trifolium repens*, the cytokinin BA also induced somatic embryos in the hypocotyl epidermis of immature zygotic embryos (Maheswaran and Williams, 1985). Recently, thidiazuron (a synthetic cytokinin) has been used to successfully induce somatic embryo formation in *Phaseolus* bean and peanut seedling cultures (Malik & Saxena, 1992a,b; Saxena et al., 1992). Further tests of somatic embryogenesis of UH965 lamina cultures using 2iP and 2,4-D indicated that 2iP alone induced somatic embryogenesis. 2iP and a low level of 2,4-D also induced somatic embryos, but to a lesser extent.

The role of cytokinins in somatic embryogenesis of anthurium needs to be studied in more detail.

Secondary somatic embryos were observed on the surface of primary somatic embryos in some of the UH1060 laminae. These secondary embryos can be detached easily with a scalpel. Secondary embryos derived from primary embryos on the hypocotyl region has been reported in many plants (Williams and Maheswaran, 1986). In anthurium (UH1060), secondary embryos could be easily detached from the primary embryo to develop a typical bipolar structure on suitable medium. When the developed somatic embryos were placed in solid medium, they formed multiple shoots, instead of single shoot, after long term culture.

Calcium increased the percentage of somatic embryogenesis in UH1060 lamina cultures when the concentration was increased from 1.5 to 2.0 mM in the medium. In carrot embryogenic suspension cultures, the higher calcium concentration also increased the number of somatic embryos (Jansen et al., 1990). However, when the calcium concentration was too high (over 6 mM), no callus or somatic embryo formed in anthurium, while increasing the calcium concentration from 6 mM and 10 mM promoted somatic embryogenesis in carrot suspension cultures (Jansen et al., 1990).

In *Renanthera* orchid, leaf culture lead to shoot bud differentiation at the leaf bases (Seeni & Latha, 1992). However, the regeneration of the orchid shoot requires both BA and NAA in the culture medium. In anthurium, a cytokinin alone (BA or kinetin) was effective in shoot regeneration from cultured laminae. When detached leaves of *Graptopetalum paraguayense* (a Crassulaceae plant) were cultured in Hoagland nutrient solution, the leaf bases could regenerate shoots. A dramatic change in growth polarity was

correlated with calcium ion (Ca^{2+}) influx and the ion current in the leaf base. The authors speculated that calcium ion influx might be involved in the initiation of organogenesis, possibly through the elevation of cytoplasmic calcium levels and activation of calcium-messenger system (Hush et al., 1991). In anthurium lamina culture, the basal part was the most responsive in producing callus and somatic embryos (on C medium) and shoots (on H1 medium) under dark conditions. Increasing calcium concentration in media also increased the percentage of somatic embryogenesis in anthurium UH1060 lamina culture. Whether there is any ion current change in the lamina culture of anthurium remains to be tested.

Intact excised laminae of UH1060 and UH965 were found to be very useful to obtain etiolated shoots when cultured in the dark. These shoots can serve as source materials for cocultivation with *Agrobacterium* and for particle bombardment and transient expression assays of reporter genes (Chapters 4 and 5).

The root regeneration system of anthurium may prove very useful for genetic transformation and for commercial micropropagation. Many roots occur in long-term cultures of *in vitro*-grown anthurium plants. For the purpose of mass propagation, the long roots can be excised and cultured, without damaging the growth of stock plants. Root culture was reported once in *A. andraeanum*, but only resulted in callus formation (Finnie & van Staden, 1986). This is the first study on root culture and shoot regeneration in anthurium. Root cuttings had been used as propagation materials for some dicot plants, such as flowering crabapple and lilac (Hartmann & Kester, 1975). Root regeneration *in vitro* is very efficient in *Arabidopsis*, which makes it a useful system for cocultivation with *Agrobacterium* and

regenerating transgenic plants in a short time (Huang & Ma, 1992; Márton & Browse, 1991; Valvekens et al., 1988). Roots were also used as an explant source for shoot regeneration of carambola (*Averrhoa carambola* L.), in which tissue culture and regeneration was very difficult (Kantharajah et al., 1992). The regeneration of shoots from anthurium root explants offers an opportunity for testing the feasibility of *Agrobacterium*-mediated transformation. This will be briefly described in Chapter 4.

In conclusion, several novel ways of anthurium regeneration through somatic embryogenesis or shoot formation using excised laminae or roots as source materials were developed. These regeneration systems may be useful for genetic transformation and micropropagation.

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CHAPTER 4

AGROBACTERIUM-MEDIATED TRANSFORMATION OF *ANTHURIUM*

4.1 INTRODUCTION

Anthurium belongs to the Araceae, one of the monocotyledonous plant families. *A. andraeanum* and its hybrids with other *Anthurium* species are used as cut flowers and flowering potted plants in Hawaii and some other countries. Due to the recent devastation of many Hawaiian anthurium farms by bacterial blight caused by *Xanthomonas campestris* pv. *dieffenbachiae* McCullock, there is a need for blight resistant cultivars (Nishijima and Fujiyama, 1985). An approach to introduce antibacterial genes into commercial anthurium is desirable to complement traditional hybridization breeding for disease resistance.

Genetic engineering of plants for bacterial disease resistance is being pursued in several crops in recent years. Many antibacterial proteins (or lytic peptides) of non-plant origin, such as insect immune proteins (Boman, 1991; Boman et al., 1991; Dunn, 1991), bacteriophage lytic proteins (Young, 1992), and animal antibacterial proteins (Lehrer et al., 1991; Ganz et al., 1990), have been isolated and characterized (Tables 2-1 and 2-2). Genes of several antibacterial proteins, including bacteriophage T4 lysozyme, chicken lysozyme, P22 phage proteins 13 and 19, cecropia insect cecropin B, and synthetic genes of cecropin B homologs, have been introduced into tobacco and potato plants (Destéfano-Beltrán et al., 1990a, b; Nagpala et al., 1990;

Düring, 1991a, b; Düring & Hippe, 1989; Hippe et al., 1989; Montanelli & Nascari, 1991).

Agrobacterium-mediated transformation has been achieved in several monocot crops (see Chapter 2). Also of relevance to anthurium transformation is a report that in vitro roots of indica rice (*Oryza sativa* L. cv. Taichung Native 1) produced transformed calli after cocultivation with *A. tumefaciens* carrying a cointegrative vector. An extract from potato suspension culture was necessary for transformation (Chan et al., 1992).

A method for tissue culture and plant regeneration in Hawaiian anthurium has been reported previously (Kuehnle & Sugii, 1991a). It is also demonstrated that somatic embryos can be induced in several media (Chapter 3), and shoot formation can be obtained from in vitro grown laminae and root explants (Chapter 3). Etiolated internodes of *Anthurium andraeanum* were shown to be transformed by wild type *Agrobacterium tumefaciens* strain C58 (Kuehnie and Sugii, 1991b). However, there is no report of anthurium transformation using engineered non-tumorigenic *Agrobacterium* and subsequent plant regeneration.

In this chapter, several methods for transformation of anthurium by *Agrobacterium tumefaciens* LBA4404 containing antibacterial gene constructs are described. Putatively transgenic anthurium plants are shown to have various degrees of resistance to the bacterial pathogen *Xanthomonas campestris* pv. *dieffenbachiae* after challenge inoculations.

4.2 MATERIALS AND METHODS

4.2.1 Plant materials and media

UH965 ('Rudolph') and UH1060 served as sources of laminae for all experiments except root cocultivation where in vitro grown plantlets of UH965, UH1003, UH1060, 'Anuenue' and 'Mauna Kea', served as sources of root explants. The parentage of UH965 and UH1060 is described in Chapter 3 (Figures 3-1B and 3-2B). Plantlets micropropagated by callus culture (Kuehnle and Sugii, 1991a) or axillary bud culture (Kunisaki, 1980) were maintained on H1 medium containing 0.2 mg/l benzyladenine (BA) (Table 4-1) in Magenta GA-7 boxes (Sigma Chem. Co., St. Louis, Missouri). Etiolated shoots, from which internode explants were derived, were obtained from cultures of either nodes or laminae on H1 medium in the dark, as described in Chapter 3, Section 3.2.7. Other culture media used for incubation and regeneration of cocultured plant materials are listed in Table 4-1. These media include Cmod (Kuehnle & Sugii, 1991a), C, F (Chapter 3) for callus formation, H1 and H2 (Kunisaki, 1980) for plant regeneration, and Su (modified from Gamborg et al., 1979) for tobacco callus (Section 4.2.5).

4.2.2 Plasmids and bacteria

Antibacterial genes were driven either by a double CaMV35S promoter (Kay et al., 1987) or by a wound-inducible promoter of potato proteinase inhibitor II (WI) (Keil et al., 1989), and ended with the NOS-3' terminal sequence (Bevan et al., 1983). The chimeric genes were in the *Hind* III site of pBI121

Table 4-1. Composition of media used in cocultivation and tissue culture of anthurium

Components	Medium and composition						
	1/2MSO	Cmod	C	F	H1	H2	Su
Macronutrients	1/2x MS	1/2x MS	1/2x MS	1/2x MS	1/2x MS	1/2x MS	MS
Micronutrients	MS	MMS[a]	MS	MS	MS	MS	MS
NaFeEDTA	25.7 mg/l	43 mg/l	25.7 mg/l	25.7 mg/l	36.7 mg/l	36.7 mg/l	43 mg/l
Vitamins	As MS[b]	As MS[b]	As MS[b]	As MS[b]	As 1/2x MS[c]	As 1/2x MS[c]	T[d]
Sucrose	30 g/l	30 g/l	20 g/l	20 g/l	20 g/l	20 g/l	40 g/l
Glucose	--	--	10 g/l	10 g/l	--	--	--
Myo-inositol	100 mg/l	--	100 mg/l	100 mg/l	100 mg/l	100 mg/l	--
2,4-D	--	0.08 mg/l	1.5 mg/l	3.0 mg/l	--	--	1 mg/l
BA	--	1 mg/l	--	--	0.2 mg/l	--	--
Kinetin	--	--	0.5 mg/l	0.5 mg/l	--	--	--
Coconut water	--	--	--	--	--	150 ml/l	--

[a]Same as MS, except H_3BO_3, $MnSO_4$ as 1/2x MS
[b]With 0.4 mg thiamine·HCl/l
[c]With 0.2 mg thiamine·HCl/l
[d]T, thiamine·HCl only, 0.1 mg/l

(Jefferson et al, 1987) (Fig. 4-1, Table 4-2). Genes encoding proteins attacin, T4 lysozyme, and P13 under control of a double CaMV 35S promoter were designated Ca2Att, Ca2T4, Ca2P13, respectively. Genes encoding proteins attacin and shiva-1 (a derivative of cecropin B) under control of WI promoter were designated WIAtt and WIShiva (Fig. 4-1). *Agrobacterium tumefaciens* LBA4404, containing the *vir*-helper plasmid (pAL4404) in Ach5 strain chromosomal background (Hoekema et al., 1983) was cultured at 28-29^{o}C on solid LB medium (10 g/l tryptone, 5 g/l yeast extract and 10 g/l NaCl, pH 7.0) (Miller, 1972) containing 25 μg/ml streptomycin (Sigma). The antibacterial gene constructs, introduced into *Agrobacterium* either by triparental mating (Ditta et al., 1980) or the freeze and thaw method (An et al., 1988), were provided by J. M. Jaynes of Louisiana State University. *Agrobacterium* with binary vectors were cultured in LB medium containing streptomycin (25 μg/ml) and kanamycin (50 μg/ml) (Clontech, Palo Alto, California). All bacteria were streak-plated on the same fresh medium every 1 to 2 months and kept at 4^{o}C. For long-term storage, bacterial suspensions were mixed with sterile glycerol (final conc. 15%) and stored at -20^{o}C for 6 months.

4.2.3 Bactericidal assay of antibacterial proteins

Four antibacterial proteins, namely attacin, cecropin B, SB37 and Shiva10 (both are cecropin B derivatives), were assayed for bactericidal activity against the anthurium blight pathogen *Xanthomonas campestris* pv. *dieffenbachiae* strain D150 (*Xcd*150) (provided by A. Alvarez, Univ. of Hawaii). These proteins were obtained from J. M. Jaynes of Louisiana State

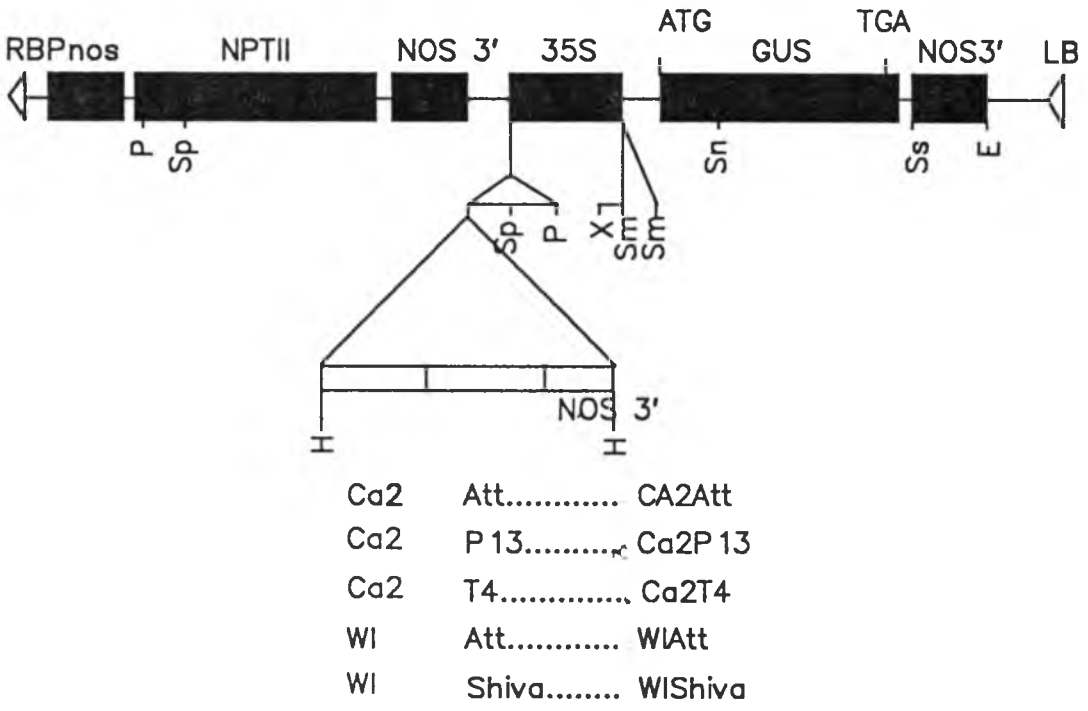

Fig. 4-1. Antibacterial gene constructs inserted into the *Hind* III site of pBI121 (Clonetech; not drawn to scale). Abbreviations: 35S, CaMV35S promoter; Ca2, double CaMV35S promoter; WI, wound inducible promoter; GUS, ß-glucuronidase; NPTII, neomycin phosphotransferase II; Att, attacin; P13, phage P22 gene *13*; T4, T4 phage lysozyme; Shiva, cecropin B analog Shiva-1; NOS3', nopaline synthase terminator; Pnos, nopaline synthase promoter; RB, right border; LB, left border; E, *Eco* RI; H, *Hind* III; P, *Pst* I; Sm, *Sma* I; Sn, *Sna* BI; Sp, *Sph* I; X, *Xha* I.

Table 4-2. Plant expression vectors carrying antibacterial genes used in transformation of anthurium

Plasmid	Properties[a]	Promoter
pCa2Att	Contains attacin F gene	Double CaMV 35S
pWIAtt	Contains attacin F gene	Wound inducible
pCa2T4	Contains T4 lysozyme *e*	Double CaMV 35S
pCa2P13	Contains phage P22 gene *13*	Double CaMV 35S
pWIShiva	Contains cecropin B analog Shiva-1	Wound inducible

[a]Chimeric genes were inserted into the *Hind* III site of pBI121 (Destéfano-Beltrán, 1991)

University, except Shiva10, which was acquired from J. M. Jaynes through R. M. Manshardt (Univ. of Hawaii). Serial dilutions of the four proteins with sterile distilled water were made and stored at -20°C until use.

The first bactericidal assay used paper filter discs (Barry & Thornsberry, 1991). Ten µl each of different dilutions (100, 50, 25 and 12.5 µg/ml) of attacin, cecropin B and SB37 were absorbed onto sterile dry filter paper discs and placed on the surface of a *Xcd*150 lawn freshly spread using a cotton swab. This produced amounts of 1, 0.5, 0.25 and 0.125 µg per disc. The supporting medium was peptone glucose agar (PDA: 1% peptone, 0.5% dextrose and 17 g/l agar), which was dispensed into 10-cm plastic petri plates. Two duplicate discs of each dilution were used in separate plates. The plates were incubated at 28°C and diameters of any inhibition zone were measured every 24 hours.

The second bactericidal assay used a microdilution method in a 96-well microtiter plate (Koneman et al., 1988). Ten µl of each protein dilution (18.75, 37.5, 75, 150, 300, and 600 µg/ml) were combined with 140 µl of bacterial suspension in each well so that the final concentrations of the four proteins were: 1.25, 2.5, 5.0, 10, 20 and 40 µg/ml, respectively. Each concentration was replicated in 3 wells. Spectrophotometric readings of log phase *Xcd*150 in 523 medium (10 g/l sucrose, 8 g/l casein acid hydrolysate, 4 g/l yeast extract, 2 g/l K_2HPO_4, 0.3 g/l $MgSO_4.7H_2O$, pH 7.0) (Kado & Heskett, 1970) were adjusted with liquid 523 medium to OD600 = 0.1, which corresponds to 2.5×10^8 colony forming units (cfu)/ml. Seven ten-fold dilutions of the bacterial suspension were made (to 10^{-7}). 140 ul of the fifth dilution (10^{-5}), which corresponds to 385 cfu when counted by spot plating a 20 µl portion onto TZC medium (Alvarez et al., 1991), was added

to each well containing proteins and to 12 lower-edge wells containing 10 ul of 523 medium (controls). 523 medium (150 ul) was also added to 12 upper-edge wells as blanks for optical density readings at 590 nm. The microtiter plate (Falcon 3072, Becton Dickinson Labware, New Jersey) was covered with a lid and incubated at 28^{o}C, on a rotary shaker (200) rpm under fluorescent light. The microtiter plate was scanned with computer-linked microplate reader (Molecular Devices Corp., Menlo Park, California) at 4, 8, 10, 12, 14 and 22 hours after the start of treatment. After this time, the plate was placed on a bench at 25^{o}C for 2 days. Optical density readings were taken one more time on the 4th day. The data were averaged and plotted against the log of the concentrations using QuatroPro software (Borland Corp., Scotts Valley, California).

4.2.4 Effect of kanamycin on formation of callus on lamina and internode explants

To determine suitable kanamycin concentrations for the selection of putative transformed cells, laminae and etiolated internode explants of UH965 and UH1060 were cultured on C medium (Table 3-1) containing 0, 25, 50, 75 and 100 ug/ml kanamycin. The number of explants that formed callus in the dark at 25^{o}C was scored after 100 days.

4.2.5 Cocultivation of explants and regeneration of anthurium plants

Individual colonies of *Agrobacterium*, each containing a different antibacterial construct (Table 4-1), were selected from a freshly subcultured

plate (within 2 weeks). For experiment 1, each colony was cultured in 5 ml liquid YEP medium (10 g/l Bacto-peptone, 10 g/l yeast extract, 5 g/l sodium chloride, pH 7.2) (An et al., 1988), containing streptomycin (25 μg/ml) and kanamycin (50 μg/ml) in a 120 ml flask and incubated on a rotary shaker (220 rpm) at 28^{o}C overnight until the solution became turbid. For colonies older than two weeks, inoculated media were shake-cultured for two to three nights. Two μl of 0.5 M acetosyringone (AS) (Aldrich Chem. Co., St. Louis, Missouri) in dimethyl sulfoxide (DMSO) were added to the overnight bacteria culture for a final conconcentration of AS at 200 μM. The bacteria plus AS mixture was diluted 10-fold with YEP medium (pH 5.5). For experiments 2 to 6, LB (pH7.0) was used for bacterial culture and dilution, instead of YEP medium, and the speed of rotation was 250 rpm instead of 220 rpm. These diluted bacteria and AS suspensions were used for cocultivation with anthurium as described in the following experiments:

Experiment 1 (initiated December 1989). Etiolated internodes of UH965 were cut with a sharp scalpel blade (#11, Feather Safety Razor Co., Japan) into 0.5-1 cm long segments, and immersed in the bacterial suspension. Internodes and *Agrobacterium* were cocultured with tobacco cell line 'Su' (Gamborg et al., 1979) as nurse culture for 5 days in the dark at 25^{o}C. Freshly subcultured 'Su' calli on Su medium (Table 4-1) were spread centrally in 10-cm plastic Petri dishes and covered with sterile Whatman #1 filter paper. The internodes and bacteria were placed side by side on top of the filter paper. Unless otherwise mentioned, all the cultures were maintained in the dark. The treated internodes were then transferred to Su medium containing 500 μg/ml of carbenicillin (Sigma) for 5 days and then

transferred to Cmod medium containing 500 μg/ml carbenicillin and 100 μg/ml kanamycin. The explants were subcultured in the same selection medium once a month for four months. They were then subcultured onto Cmod medium containing either 250 μg/ml cefotaxime (Sigma) or 500 μg/ml carbenicillin (Sigma), depending on the availability of antibiotics, and 50 μg/ml kanamycin in monthly or bimonthly intervals for a period of four months. They were subcultured on F medium containing cefotaxime (250 μg/ml) and kanamycin (50 μg/ml) for one month, then in liquid D medium containing 50 μg/ml kanamycin for shake culture at 100 rpm, 25°C for two months. To regenerate shoots from calli, they were transferred to liquid CK7 medium (Chapter 3, Section 3.2.5) containing 0.2 mg/l BA and 3 mg/l 2iP for shake culture at 100 rpm, 25°C, under light (22 $\mu Em^{-2}sec^{-1}$, 16 hours light/8 hours dark cycle). After two months, all regenerated shoots were transferred to solid H1 medium containing 50 μg/ml kanamycin in GA-7 boxes under light conditions (32 $\mu Em^{-2}sec^{-1}$, 16 hours light/8 hours dark cycle) in a tissue culture room. Rooted shoots continued to be cultured in H1 medium containing 50 μg/ml kanamycin with monthly or bimonthly subculture. At this concentration, kanamycin-sensitive plants were not easy to distinguish from resistant plants, therefore they were changed to hormone-free H2 medium containing 75 μg/ml kanamycin to allow the growth of transformed plantlets.

Experiment 2 (initiated April 1990). Two replications of twenty etiolated internodes each of what was thought to be UH1060 were cocultivated with *Agrobacterium* carrying the DNA constructs pCa2Att, pCa2P13, pCa2T4, pWIAtt and pWIShiva (Table 4-2). For controls, 20 internodes were

immersed in liquid LB medium only. All the explants were placed in 10-cm plastic Petri dishes, 20 internodes per plate, on 1/2MSO medium (Table 4-1). The cocultivation was for 3 days. Internodes were transferred to Cmod medium containing 250 ug/ml cefotaxime and 100 μg/ml kanamycin at monthly intervals for 3 months. Explants were then subcultured in the dark on H1 medium containing 50 μg/ml kanamycin. Calli with etiolated shoots were subcultured at 1- to 2-month intervals in the fresh regeneration medium with kanamycin (50 μg/ml) in Petri dishes. Two years after the initial co-cultivation, all etiolated shoots were transferred into GA-7 boxes containing H1 medium and kanamycin (50 μg/ml), and were moved into the light (32 $\mu Em^{-2}sec^{-1}$, 16 hours light/8 hours dark cycle) to recover green shoots. After one month, the green shoots were identified as UH965 instead of UH1060, based on the appearance of the leaf. The green shoots formed roots under these conditions. Plantlets were then transferred onto H2 medium containing 75 μg/ml kanamycin.

Experiment 3 (initiated September 1990). Detached laminae of UH965 and UH1060 were halved along the middle veins and then pre-cuitured in F medium (3 mg/l 2,4-D and 0.5 mg/l kinetin, Table 4-1) for 38 to 45 days before cocultivation. Half-laminae of UH965 and UH1060 were cocultured for 3 days with *Agrobacterium* carrying various plasmids, the same as in Experiment 2 (Table 4-2). The number of explants per treatment ranged from 17 to 21. Controls received only LB treatment. The explants were then transferred to C medium (Table 4-1) containing 500 μg/ml carbenicillin and 100 μg/ml kanamycin. They were subcultured on fresh selection medium three times during the next 11 months. During this period, UH965 explants

browned and were discarded without further observation. The explants of UH1060 were subcultured three times during 5 months on C medium except kanamycin was reduced to 50 μg/ml. After this, calli were grown for 5 months in the dark on H1 medium containing 50 μg/ml kanamycin. All calli were transferred to light conditions (32 $\mu Em^{-2}sec^{-1}$, 16 hours light/8 hours dark cycle) at 25°C to recover green shoots. All rooted shoots were cultured on H2 medium containing 75 μg/ml kanamycin.

Experiment 4 (initiated December 1990). In vitro-grown intact laminae of UH1060 were excised from plantlets and cocultured with *Agrobacterium* carrying 5 different antibacterial constructs as described in experiment 2. The cocultures were incubated on modified H1 medium (containing 0.5 mg/l kinetin instead of 0.2 mg/l BA, designated 0.5K) for 5 days until bacterial growth was observed. The number of explants per treatment ranged from 60 to 63 per four plates. After co-cultivation the laminae were transferred to 0.5K medium containing 500 μg/ml carbenicillin and 100 μg/ml kanamycin. Twenty-five days after initial cocultivation, the cultures were divided into two parts, each with 2 plates. Two plates of each treatment were moved to low light conditions (4 $\mu Em^{-2}sec^{-1}$, 16 hours light/8 hours dark cycle) at 25°C. The other 2 plates were incubated in the dark at 22-25°C. After 1 month, the samples forming small calli were sequentially transferred onto the C, H1, C, Cmod, C, and H1 media in the dark, with periods ranging from 1 to 3 months per medium for a total elapsed time of 9 months. All media contained carbenicillin (500 μg/ml) and kanamycin (50 μg/ml). Etiolated shoots regenerated from calli were then transferred to H1 medium with 50 μg/ml kanamycin for about 5 months at one time and kept in the dark. The

etiolated shoots and some of the shooted calli were then transferred onto H2 medium (Table 4-1, Kunisaki, 1980) containing 75 μg/ml kanamycin and cultured in the light conditions (32 $\mu Em^{-2}sec^{-1}$, 16 hours light/8 hours dark cycle) at 25°C to recover green shoots. Total time period from initial cocultivation to green shoots was about 13 months.

Experiment 5 (initiated August 1991). The effect of tobacco nurse cells on transformation of anthurium was studied. Tobacco cell line 'Su' (Gamborg et al., 1979) was maintained on Su medium as a friable callus (Table 4-1). To serve as a nurse culture, tobacco callus was subcultured on fresh Su medium and used for cocultivation within 4 days of each subculture. Internode segments of UH965 and UH1060 from etiolated shoots were cut with a sharp No. 11 blade into pieces 0.5-1 cm in length. *Agrobacterium tumefaciens* LBA4404/pCa2Att was cultured overnight in LB medium containing 50 μg/ml kanamycin and 25 μg/ml streptomycin. AS was added to the bacterial suspension to a final concentration of 200 μM. The AS solution (containing bacteria) was diluted 10-fold with LB medium. Internodes were cocultured for 3 days with diluted AS and bacteria solution on top of a plate with tobacco 'Su' cells as nurse culture, or on Cmod medium without 'Su' nurse. 25 to 26 internodes were placed in 10-cm plastic Petri plates, two plates per treatment (nurse vs. no nurse, pCa2Att and LB control). After cocultivation, all explants were transferred to Cmod medium containing 500 μg/ml carbenicillin and 50 μg/ml kanamycin. They were subcultured on fresh Cmod selection medium three times in about 3 months. Explants were then transferred to H1 regeneration medium containing kanamycin (50 μg/ml) and either 250 μg/ml cefotaxime or 250

μg/ml carbenicillin. Cultures were incubated in low light (4 $\mu Em^{-2}sec^{-1}$, 16 hours light/8 hours dark cycle) at 25°C. UH965 samples continued to be subcultured monthly on H1 medium with antibiotics until green shoots regenerated. Under the same conditions, UH1060 callus grew slower than UH965, so C medium with similar antibiotics was used once for 1 month during regeneration. Regenerated shoots were easily removed from callus. Some shoots formed roots at this time. The shoots or plantlets were transferred to H2 medium with 75 μg/ml kanamycin. They were incubated in higher light (32 $\mu Em^{-2}sec^{-1}$, 16 hours light/8 hours dark cycle) for shoot growth and root formation. Under this higher light condition, plantlets were considered kanamycin-sensitive if a newly developed leaf was bleached. In one case of bacterial regrowth (only one box of UH1060), plantlets were transferred to H2 medium with both carbenicillin and kanamycin to kill the bacteria and maintain the selection condition.

Many kanamycin-resistant shoots of UH965 and UH1060 were transferred to H1 medium containing kanamycin for multiplication in low light (4 $\mu Em^{-2}sec^{-1}$, 16 hours light/8 hours dark cycle). Rooted plantlets were transferred to H2 medium with 75 μg/ml kanamycin in higher light (32 $\mu Em^{-2}sec^{-1}$, 16 hours light/8 hours dark cycle). In other cases, shoots regenerated from calli were directly transferred to H2 medium containing 75 μg/ml kanamycin to screen out under higher light conditions any ambiguous (unclear bleaching) kanamycin-sensitive plantlets.

Experiment 6 (initiated November 1991). Roots from in vitro- grown plantlets of UH965, UH1003, UH1060, 'Anuenue' and 'Mauna Kea' were cut into pieces 0.5-1 cm in length and cocultivated for 24 hours with diluted

solution of AS and *Agrobacterium tumefaciens* LBA4404 carrying pCa2Att (same as experiment 2). Controls were treated with LB only. Root explants were transferred to H1 medium containing 250 μg/ml cefotaxime and 50 μg/ml kanamycin and incubated at 23-25°C for 2 months. They were then transferred to the same selection medium except the kanamycin concentration was reduced to 25 μg/ml due to the slow growth of induced callus. After 5 months, the callused root explants were transferred to C medium (Chapter 3, Table 3-1) containing 250 μg/ml carbenicillin and 50 μg/ml kanamycin and incubated at 25°C in low light (4 $\mu Em^{-2}sec^{-1}$, 16 hours light/8 hours dark cycle) for one week. Thereafter, the green callus was transferred to fresh H1 medium with antibiotics. The regenerated shoots along with calli were transferred to H2 medium containing carbenicillin and 75 μg/ml kanamycin. The number of explants with yellow callus was counted 7 months after initial cocultivation. Some of the yellow calli turned to green under low light after 7 months. These green calli were counted 11 months after initial cocultivation.

4.2.6 GUS histochemical staining

Leaf and root segments from kanamycin-resistant plantlets were excised and stained for GUS activity. Two kinds of GUS buffers were used interchangeably, depending on the availability. The first buffer consists of 10 mM Na_2-EDTA, 100 mM $NaH_2PO_4 \cdot H_2O$, 0.5 mM $K_4Fe(CN)_6 \cdot 3H_2O$ and 0.1% Triton X-100 (McCabe et al., 1988), pH 7.0. The second buffer was provided by G. L. Nan (Univ. of Hawaii, Honolulu, Hawaii). This buffer was developed by J. Yoder of Univ. of California at Davis. It consists of

100 mM sodium phosphate, pH 7.0, 1% Triton X-100, 1% dimethyl sulfoxide (DMSO), and 10 mM Na_2-EDTA.

5-Bromo-4-chloro-3-indolyl-ß-D-glucuronic acid (X-gluc) (Research Organics, Cleveland, Ohio) was dissolved in DMSO (50 mg/ml) and stored at -20°C until use. For detection of GUS activity in anthurium tissues, a solution (reaction buffer) of 50 µl of X-gluc/DMSO in 5 ml GUS buffer was freshly prepared.

The leaf and root segments of untreated controls and kanamycin-resistant plantlets were incubated in reaction buffer at 37°C for 2 days. To visualize the blue color, 1 ml of 95% ethanol was added to each sample in a 1.5 ml microcentrifuge tube and incubated at 25°C with occasional shaking until chlorophyll was absent in the leaf segments. The samples were examined both with and without use of dissecting microscope.

4.2.7 De novo callus induction from lamina culture of regenerated kanamycin-resistant anthurium

Laminae from kanamycin-resistant plantlets of UH965, regenerated following tissue co-cultivation with *Agrobacterium tumefaciens* carrying pCa2Att, were excised and cultured on C medium containing 50 µg/ml kanamycin in the dark at 25°C to induce callus. A total of 147 lamina from 15 GA-7 boxes were cultured in 15 plastic petri plates. These calli served as source material for protein extraction.

4.2.8 DNA isolation

Initial DNA extraction was according to Dellaporta et al. (1983), as described in Herrera-Estrella and Simpson (1987), with minor modification. About 0.5-1.2 g of in vitro leaf tissues were frozen in dry ice powder and ground to a fine powder. The tissue powder was mixed with 15 ml of extraction buffer (100 mM Tris-HCl, pH 8, 50 mM EDTA, pH 8, 500 mM NaCl, and 10 mM ß-mercaptoethanol). The extract was mixed thoroughly with 1 ml of 20% SDS and incubated in a 65^{o}C water bath for 10 minutes. Five ml of 5 M potassium acetate (Fisher, Pittsburgh, Pennsylvania) was thoroughly mixed with the extract and incubated on ice for 30 minutes. The mixture was spun at 25,000 x g (Sorval SS34 rotor, 14,500 rpm) in a Sorvall refrigerated high-speed centrifuge (RC-5B, DuPont, Wilmington, Delaware) for 20 minutes at 4^{o}C. The supernatant was filtered through a Miracloth filter (Calbiochem, La Jolla, California) and mixed with 10 ml of ice-cold isopropanol (Fisher). The mixture was incubated at -20^{o}C for at least 30 minutes and centrifuged at 20,000 x g for 30 min at 4^{o}C. The pellet containing nucleic acids was washed with 2 ml of absolute ethanol to remove chlorophyll. The clarified pellet was dissolved in 300 μl TE buffer, pH 8.0 (10 mM Tris·HCl, 1 mM EDTA, pH 8.0) (Maniatis et al., 1982). The centrifuge tube was rinsed once with 100 μl TE. Both solutions were combined and transferred into a 1.5 ml microfuge tube. Five μl of DNase-free RNase (5 mg/ml) (Sigma) was added to the solution and incubated at 37^{o}C for 30 minutes. The solution was extracted twice with equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma). The aqueous phase was extracted once with equal volume of chloroform-isoamyl alcohol (24:1).

The volume of the aqueous phase was adjusted to 400 µl with TE buffer and 40 µl of 3 M sodium acetate was added to the solution and mixed well. Absolute ethanol (2.5 volumes) was added to the mixture and mixed well. The solution was incubated at -20°C for 30 minutes and spun in the Eppendorf Model 5402 refrigerated microcentrifuge at top speed (14,000 rpm) at 4°C for 30 minutes. The DNA pellet was washed twice with 70% ethanol. The pellet was air-dried for 5 minutes and resuspended in 100 to 300 µl TE buffer. Portions of diluted DNA were measured at OD260 in a spectrophotometer (Milton Roy Spectronic 601) to calculate concentration. The ratio of OD260 to OD280 indicates the purity of the DNA solution. Using this modified Dellaporta method, the ratio usually ranged above 1.70. The DNA solutions were stored at -20°C until use.

4.2.9 DNA analysis using PCR

Genomic DNA isolated from leaves of untreated and kanamycin-resistant anthuriums and plasmid DNA were used for PCR amplification of specific target genes. The primers (0.25 µg each) for *gus* (Jefferson et al., 1986), *nptII* (Chee et al., 1989; Beck et al., 1982), *Att* (Kockum et al., 1984), *P13* (Rennell and Poteete, 1985), and T4 lysozyme (Owen et al., 1983) genes are listed in Table 4-3. A shorter 3' primer of *nptII* gene (Table 4-3) was used later in some PCR analyses. Taq DNA polymerase (1.25 units/50 µl reaction) and 10X buffer with magnesium ion were from Promega (Madison, Wisconsin) and used according to manufacture's direction. Final concentration of dNTPs (dATP, dCTP, dGTP and dTTP; Fisher) was 200 µM each. The samples were amplified in a Coy TempCycler model 60 (Coy

Table 4-3 Nucleotide sequence of primers used in PCR amplification

Primer	Sequence	Tm[a]	Position on gene
GusA 5'	5'-GCATTCAGTCTGGATCGCGA-3'	62	357-376
GusA 3'	5'-TCACCGAAGTTCATGCCAGT-3'	60	2061-2080
NPTII 5'	5'-CCCCTCGGTATCCAATTAGAG-3'	64	531-551[a]
NPTII 3'-1	5'-CGGGGGGTGGGCGAAGAACTCCAG-3'	82	1562-1585[b]
NPTII 3'-2	5'-GTGGGCGAAGAACTCCAGCAT-3'	66	1559-1579[b]
Att 5'	5'-ATGGACGCGCACGGAGCCCTT-3'	70	1-18[c]
Att 3'	5'-TCCGAAGTTAGGCTCCCAAGA-3'	64	523-543
P13 5'	5'-ATGCCAGAAAAACATGATCTG-3'	58	65-85
P13 3'	5'-TGCTGATTTGCATCATCGAC-3'	58	358-377
T4 5'	5'-ATGTTACGTATAGATGAACGTA-3'	58	16-37
T4 3'	5'-CAGTTCTAAACGTTGTAATGAC-3'	60	444-465

[a]Tm (melting temperature) = 4 x (G+C) + 2 x (A+T)
[b]In P_{NOS}NPTII gene of pGA482 (Chee et al., 1989)
[c]Starts from pCP521 first amino acid except ATG codon was absent in original sequence (Kockum et al., 1984)

Corporation, Grass Lake, Michigan) with the following conditions: one cycle of denaturation at 94°C for 3 minutes, then 29 cycles at 94°C for 1 minute, 56° (or 58°, or 60°C, depending on the melting temperature, Tm, of specific primers) for 1 minute, and 72°C for 2 minutes. In the last cycle, the reaction at 72°C was extended to 9 minutes. After PCR amplification, 10 μl (amplified from plant DNA) or 2-5 μl (amplified from plasmid DNA) of each reaction were run in 0.7 or 0.8% agarose gel (Fisher Low EEO) in 0.5X TBE buffer (1X TBE: 0.089 M Tris-borate, 0.089 M boric acid, 0.002M EDTA, pH 8.0) (Maniatis et al., 1982) at 47 or 58 volts. After electrophoresis, the gel was stained with ethidium bromide and visualized under UV light. The amplified bands were photographed with Polaroid 665 or 667 film using a Polaroid MP4 camera (Cambridge, Massachusettes).

4.2.10 Southern blot analysis

Genomic DNA (10 μg each) from untreated control and kanamycin-resistant UH1060 Ca2P13 plantlets was digested separately with restriction enzymes *Hind* III, *Eco* RI and *Sma* I (Promega) according to manufacturer's directions. The digestion was performed at 37°C (*Hind* III and *Eco* RI) and 30°C (*Sma* I) for 14-16 hours. After digestion, DNAs were separated on 0.6% agarose (Fisher Low EEO) in 0.5X TBE buffer at 47 volts. The gel was stained with ethidium bromide and photographed.

The gel was denatured in 0.2 N NaOH and 0.6 M NaCl for 30 minutes. It was then neutralized with 0.5 M Tris/1.5 M NaCl, pH 7.5, for 30 min. DNA was blotted onto Zetabind nylon membrane (pore size 0.45 μm; CUNO, Inc., Meriden, Connecticut) by capillary action (Southern, 1975) with 20X SSC

(3 M sodium chloride, 0.3 M sodium citrate, pH 7.0) overnight. The blotted membrane was washed twice in 2X SSC for 15 minutes each and dried at 28^{o}C until use. The gels containing PCR amplified and separated DNA fragments were processed in the same way for genomic DNA blot.

The dried membrane was prehybridized in a sealed plastic bag containing 20 ml hybridization solution (5X SSC, 1% blocking reagent, 0.1% (w/v) N-lauroylsarcosine Na-salt, and 0.1% SDS) (Genius Nonradioactive DNA Labeling and Detection Kit, Boehringer Mannheim, Indianapolis, Indiana) at 68^{o}C for 6 hours to overnight. For probe preparation, *Att* and *P13* were amplified from separate plasmids, pCa2Att and pCa2P13, respectively, with specific primers (Table 4-3) by polymerase chain reaction (PCR) as described in section 4.2.9. The amplified DNA fragments were separated on 0.8% agarose gel and the corresponding band cut out and purified. The DNA fragments were labeled with digoxigenin-11-dUTP by the random priming method (Genius Kit). Probes were freshly denatured by boiling for 5 min and cooled on ice. The DNA blot was sealed in a plastic bag and prehybridized in hybridization buffer at 68^{o}C for 6 hours to overnight. The prehybridized DNA blot was then incubated with hybridization solution and probe. The hybridization was at 68^{o}C for 14 to 16 hours. After hybridization, the membrane was washed twice with 2X SSC and 0.1% SDS at 25^{o}C for 5 min each. The membrane was then washed twice with 0.1X SSC and 0.1% SDS at 68^{o}C for 15 min each.

Color detection of the hybridized membrane was according to manufacturer's directions (Genius Kit, Boehringer Mannheim). This involved blocking the washed membrane with 1% blocking reagent at 25^{o}C for 30 min. The membrane was then incubated for 30 minutes with anti-

digoxigenin antibody conjugated with alkaline phosphatase. Color was detected by immersing the membrane in color-developing solution and 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium salt (NBT). In some cases, the membrane was reacted instead with a Lumi-Phos 530 (Boehringer Mannheim) solution and exposed to Kodak XAR-5 film for 10 seconds (DNA blots from PCR) to 30 minutes (genomic DNA blot) at 25^{o}C.

4.2.11 Western blot analysis

Total protein was isolated from de novo calli formed on lamina cultures of untreated plants on C medium or from kanamycin-resistant regenerated plants on C medium containing 50 μg/ml kanamycin. Calli were frozen at -20^{o}C and ground in 2 volumes of ice-cold 1X phosphate buffered saline (PBS) buffer (5 Prime-3 Prime, Boulder, Colorado). The extracts were centrifuged at top speed in an Eppendorf microcentrifuge at 4^{o}C for 10 minutes. The supernatants were transferred to 1.5-ml microcentrifuge tubes. In the analysis for T4 lysozyme expression, leaf tissue was used for protein extraction. The protein concentration was determined by the Lowry method (Lowry et al., 1951) using BSA as a protein standard.

To run SDS-PAGE, samples of 30 or 60 μg total protein were mixed with 2X sample buffer (0.25 M Tris·HCl, pH 6.8, 4% SDS, 2% ß-mercapto-ethanol, 20% glycerol, 3 mg/100 ml bromophenol blue) (recipe from Hoefer Scientific Instruments, San Francisco, California) and boiled in a water bath for 2 minutes. Cooled protein samples were loaded into the wells of a 12% SDS-polyacrylamide gel in a Hoefer SE280 apparatus (Hoefer Scientific

Instruments) or Mini-Protean II (Bio-Rad, Richmond, California). A protein molecular weight standard (Bio-Rad, LMW) was added in the side wells for molecular weight estimation. Partially purified cecropia insect attacin (4.5 μg, provided by J. M. Jaynes) and IPTG-induced T4 lysozyme from expression vector pHSe5 (Muchmore et al., 1989; provided by F. W. Dahlquist through A. Roth, Institute of Molecular Biology, Univ. of Oregon) in *E. coli* DH5α cells were also loaded in separate wells of different gels for comparison. The gel composition and all the buffers were prepared according to the recipes provided by Hoefer. Each gel was run at 150 volts at 25^{o}C for 45 minutes to 1.5 hour. The proteins were electroblotted onto a nitrocellulose (NC) filter (Bio-Rad) with transfer buffer (18.75 mM Tris, 144 mM glycine, 20% v/v methanol, pH 8.3) in the Mini Trans-Blot system (Bio-Rad). The transfer procedure was according to the manufacturer's directions. The time for transfer was 1 hour at 250 mA constant current in an ice-cooling box. After electroblotting, one of the edge wells containing molecular weight markers was cut out and stained with 1X Amido Black solution (Sigma) for 1 minute and destained in 15% isopropanol and 10% acetic acid in water for 30 minutes. The remaining NC filter was processed for immunodetection.

The procedure for immunodetection of attacin and T4 lysozyme proteins was basically the same as described in the Immun-Blot Assay (Bio-Rad), except that the time and buffer volume were changed as follows. The NC filter was blocked with Tris buffered saline (TBS)-3% gelatin for 45 minutes at room temperature. It was washed with TTBS (TBS with 0.05% Tween-20) buffer for 5 minutes. Rabbit anti-attacin antibody (provided by J. M. Jaynes, Louisiana State University) was diluted 400-fold in 5 ml antibody

buffer (1% gelatin in TTBS) and applied to the NC blot in a plastic bag for 60 minutes at room temperature. Crude T4 lysozyme antibody prepared by Ron Kaback was provided by A. Roth (Institute of Molecular Biology, Univ. of Oregon). The filter was washed for 15 minutes twice with 100 ml TTBS. Secondary antibody (mouse anti-rabbit IgG) conjugated with alkaline phosphatase (Bio-Rad) was diluted 3000-fold in 5 ml antibody buffer (1% gelatin in TTBS) and incubated with the filter for 40 minutes. The unbound antibody conjugate was removed by washing twice with TTBS for 10 minutes each. The detergent Tween-20 was removed by washing with 100 ml TBS for 5 minutes. Protein bands were detected by color reaction of the filter in a color development buffer and buffer system (Bio-Rad) containing substrate for alkaline phosphatase, X-phosphate, and oxido-reduction agent NBT. The color development was stopped by immersing the filter in distilled water for 10 minutes.

4.2.12 Challenge of kanamycin-resistant anthurium with *Xanthomonas campestris* pv. *dieffenbachiae*

Kanamycin-resistant anthuriums from the pCa2Att-treated experiment and untreated anthurium plantlets from tissue culture were transplanted into 5-inch plastic pots containing tree-fern fiber. Plants were grown in the laboratory under 14 hours light/10 hours dark cycle. The temperature of the lab ranged from 22^{o} to 25^{o}C. Two- month-old plantlets were used for the first bacterial challenge study. The most recently matured leaf lamina from each plantlet was cut off by a scalpel blade. Immediately after lamina excision, 2 μl containing an average of 25 colony forming units (cfu) of

Xanthomonas campestris pv. *dieffenbachiae* strain D150 (*Xcd*150, a virulent strain isolated from Hilo, streptomycin-resistant, serotype 5, provided by A. Alvarez, Univ. of Hawaii) was applied onto the exposed petiole ends. After inoculation of petioles with bacteria, each pot was wrapped with a plastic bag and incubated at 25^{o}C for 24 hours. The inoculated plantlets were cultured in the lab conditions described above for 20 days without the plastic bags. The plantlets were transferred to a growth chamber with 14 hours light/10 hours dark cycle, 28^{o}C and 71% relative humidity. After three weeks, the symptoms were recorded.

In a second trial of challenge inoculation, greenhouse-grown kanamycin-resistant anthuriums from pCa2Att, pCa2P13 and pCa2T4-treated experiments (one pot of each type, 8-12 plants per pot) and untreated control plants were inoculated with an average of 24 cfu per 2 μl of *Xcd150* to the petiole cut ends. The symptoms were recorded and reisolations of the bacteria were performed after 4 weeks of incubation in a growth chamber with similar conditions (14 hours light/10 hours dark cycle, 28^{o}C) except for no humidity control.

4.2.13 Reisolation of inoculated bacteria

To check if the challenged plants contained *Xcd*150, not other strains, the inoculated petiole and stem base from each plantlet was cut into 0.5 cm sections for reisolation of the bacteria. For the first batch, three infected untransformed plants, two infected kanamycin-resistant plants and one healthy kanamycin-resistant plant were used for bacterial reisolation. The tissue sections were cut into halves and immersed in 150 μl saline solution

(8.5 g/l sodium chloride in sterile water) in a 96-well microtiter plate for one hour. Five 20-μl aliquots of the bacterial suspension were spot plated onto semiselective esculin-trehalose medium (ET) (Alvarez et al., 1991; Norman & Alvarez, 1989) containing 200 μg/ml cycloheximide in 10-cm Petri plates and incubated at 28^{o}C for two days. The bacterial micro-colonies from each spot were counted using a dissecting microscope.

In the second batch, three tissue sections (top, middle and base parts of petioles) of every remaining plant were repeated for bacterial isolation on ET medium containing 400 μg/ml cycloheximide (to control fungal growth). The bacteria from every tested plant were restreaked on tetrazolium chloride (TZC) medium (Alvarez et al., 1991; Norman & Alvarez, 1989) containing streptomycin. TZC medium was used to examine the uniformity of the colony types, which is a check of the purity of the strain (Norman & Alvarez, 1989). The plates were incubated at 28^{o}C for 2 days. Selected bacterial colonies were verified by an enzyme-linked immunosorbent assay (ELISA) test using specific monoclonal antibodies Xcd108, Xcd1, Xcd3, Xcd7, Xcd30, Xcd47, and Xcd108 (Alvarez et al., 1991) (provided by A. Alvarez of Plant Pathology, Univ. of Hawaii) using the original pathovar *Xcd*150 for comparison.

In the second challenge experiment, only the top, middle and base parts of the bacteria-inoculated petiole were used for bacterial reisolation. Only selected colonies were restreaked onto TZC medium to check for colony purity.

4.3 RESULTS

4.3.1 Bactericidal activity of antibacterial proteins

In the first bactericidal assay, no inhibition zones were observed after 24 and 48 hours of culture of *Xcd*150 around paper filter discs containing various concentrations of antibacterial proteins (attacin, cecropin B and SB37).

In the microdilution assay, in the first 22 hours of bacterial culture in 523 medium, none of the antibacterial protein treatments showed measurable growth. When the microtiter plate containing the bacteria was then placed on a bench at 25^{o}C for 3 days, a cloudy growth of the bacterial suspension was observed in all control wells with *Xcd*150 except one edge well. Treatments with relatively low concentrations of antibacterial proteins also had cloudy growth: attacin at 1.25 to 5 μg/ml; cecropin at 1.25 and 2.5 μg/ml; SB37 at 1.25 μg/ml (Fig. 4-2, picture and optical density measurement taken at fourth day). All other treatments showed clear solutions in the wells; of note were the wells of Shiva10 which were clear at all dilutions (Fig. 4-2). These results indicate that the antibacterial proteins are effective against *Xcd*150 growth. Based on this assay, the minimal concentrations of antibacterial proteins inhibiting bacterial growth are: attacin, 10 μg/ml; cecropin B, 5 μg/ml; SB37, 2.5 μg/ml; and Shiva10, 1.25 μg/ml (Fig. 4-3).

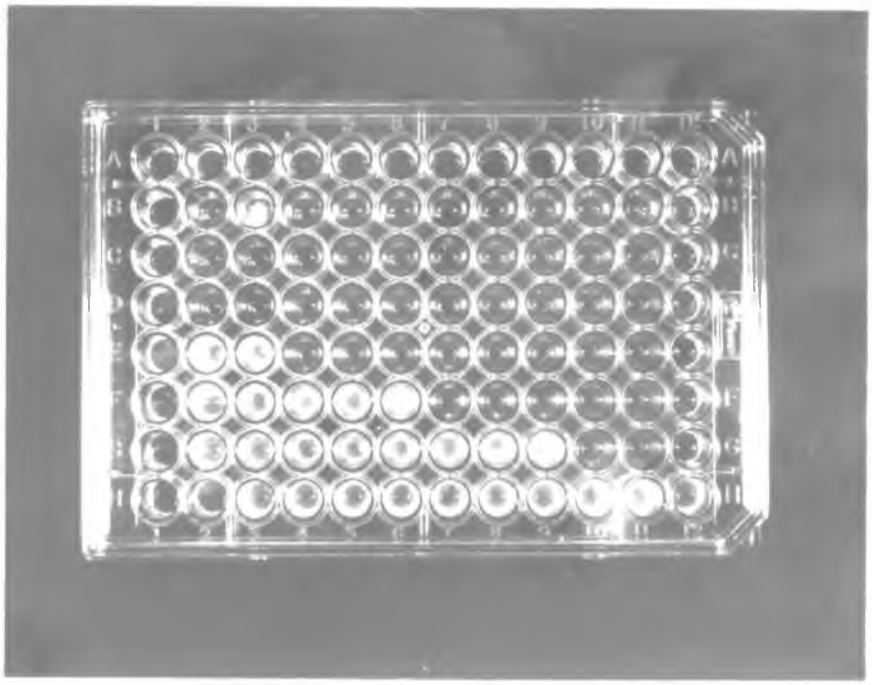

Fig. 4-2. Bactericidal assay of antibacterial proteins attacin, cecropin B, SB37 and Shiva10 on *Xanthomonas campestris* pv. *dieffenbachiae* strain 150 by a microdilution method. Each protein dilution has 3 replications. Row A, 523 medium only; Rows B-G, dilutions of proteins from 40, 20, 10, 5, 2.5, and 1.25 μg/ml, respectively; Row H, *Xcd*150 and 523 medium; Columns 1-3, attacin; 4-6, cecropin B; 7-9, SB37; 10-12, Shiva10. Picture was taken four days after assay. Cloudy growth in well B3 was due to fungal contamination after the assay. The edge wells are not clear due to photographic effect.

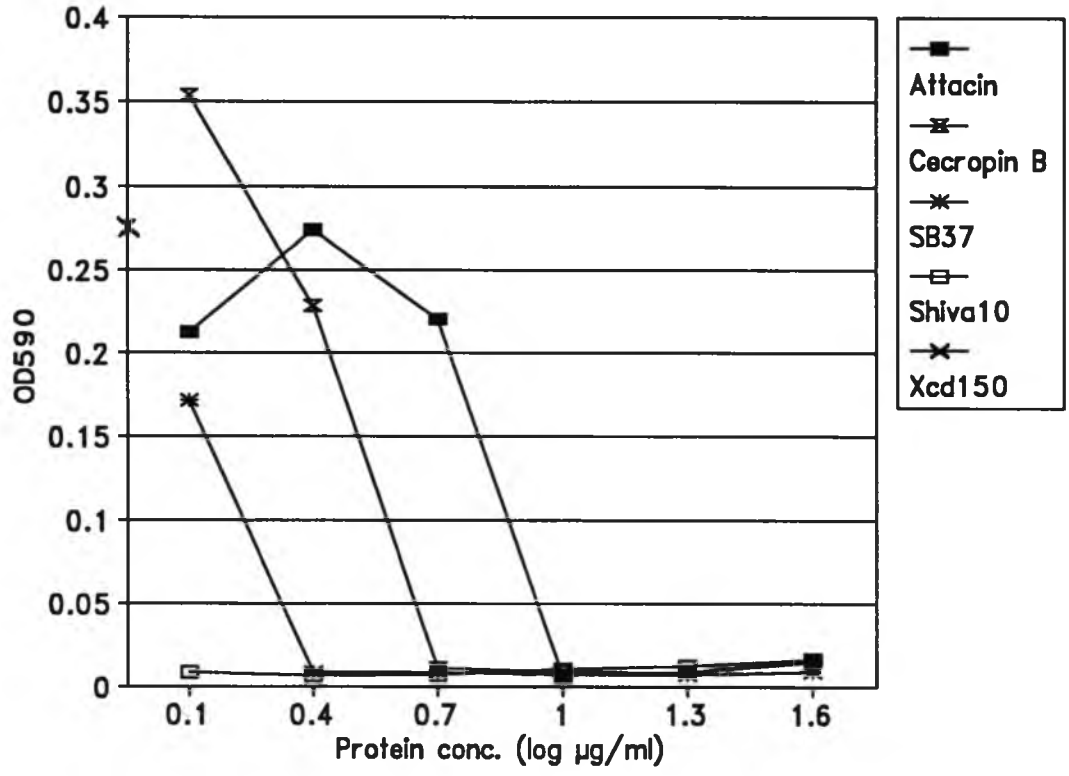

Fig. 4-3. Optical density changes of *Xanthomonas campestris* pv. *dieffenbachiae* strain D150 (Xcd150) affected by different concentrations of antibacterial proteins, attacin, cecropin B, SB37 and Shiva10. Bacteria were cultured in 96-well microtiter plate. Data were measured 4 days after culture. Each data point is the average of three replications, except Xcd150 control, which is the average of 12 replications.

4.3.2 Optimal kanamycin concentration for selection

In UH965 lamina cultures, 22.5% (no. responding/total) of explants formed callus on medium lacking kanamycin, while 60% (no. responding/total) formed callus in medium containing 25 μg/ml kanamycin. The appearance of calli formed on medium with 25 μg/ml kanamycin is the same as on medium without kanamycin. Higher kanamycin concentrations inhibited callus formation (Table 4-4). In internode cultures of UH965, 70% formed callus in the medium lacking kanamycin, 5% formed callus in the medium with 25 μg/ml kanamycin, and 15% formed callus in the medium with 50 μg/ml kanamycin (Table 4-4). No callus was observed on higher kanamycin concentrations.

In UH1060 lamina cultures, 100% of the explants formed callus in the absence of kanamycin. In the presence of 25 μg/ml kanamycin, 12.5% of the explants formed callus. At higher kanamycin concentrations, callus formation was inhibited (Table 4-4). In internode cultures of UH1060, 12.5% formed callus in the absence of kanamycin. Only 2.5% of the explants formed callus in the presence of 25 μg/ml kanamycin. The calli formed in the presence of 25 μg/ml kanamycin were smaller than in the absence of kanamycin. Higher concentrations of kanamycin are also inhibitory to callus formation in internode cultures (Table 4-4).

Table 4-4. Effect of kanamycin on callus formation of UH965 and UH1060 lamina and internode cultures in C medium after 100 days

Cultivar	Kanamycin (μg/ml)	No. lamina cultured	No. forming callus (%)		No. internode cultured	No. forming callus (%)	
UH965							
	0	40	9	(22.5)	40	28	(70)
	25	40	24	(60)	40	2	(5)
	50	40	0	(0)	40	6	(15)
	75	40	0	(0)	40	0	(0)
	100	40	0	(0)	40	0	(0)
UH1060							
	0	40	40	(100)	40	5	(12.5)
	25	40	5	(12.5)	40	1	(2.5)
	50	40	0	(0)	40	0	(0)
	75	40	0	(0)	40	0	(0)
	100	40	0	(0)	40	0	(0)

4.3.3 Callus formation and regeneration of UH965 and UH1060 after cocultivation

In Experiment 1, some internodes of UH965 formed callus in one month in all treatments, including the controls which were not treated with *Agrobacterium* (Table 4-5). The percentage of internodes with callus for each treatment was 40% for control, 95% for pCa2Att, 80% for pCa2P13, 65% for pCa2T4, and 80% for pWIAtt, respectively. The treatment with pWIShiva was contaminated by fungus during cocultivation, and was not included in scoring. The callus in the controls was smaller than in all other treatments. Besides, the calli of the controls did not have the yellow, compact appearance of calli on *Agrobacterium*-treated internodes. After 4.5 months of culture in the dark, internodes in three of the treatments produced very small shoots from the callus. The percentage of explants with shoots was 20% for pCa2Att, 5% for pCa2P13, and 10% for pCa2T4 (Table 4-5). Controls did not form shoots. For the subsequent 6-8 months, shoot growth was slow. These shooted calli were transferred into light in liquid CK7 medium. Regenerated green shoots along with calli (Fig. 4-4) were then transferred to solid medium containing kanamycin. In 1.5 years after cocultivation, kanamycin-resistant, or putative kanamycin-resistant, shoots with roots were recovered from treatments with *Agrobacterium* carrying plasmids pCa2Att, pCa2P13 and pCa2T4. At least 300 kanamycin-resistant plants were transferred into potting mix containing tree-fern fibers. They were grown in the greenhouse for future evaluation of plant performance (i.e., bacterial disease resistance, flower characters). The percentage of kanamycin-resistant UH965 plants regenerated from

Table 4-5. Response of anthurium UH965 internodes cocultivated with *Agrobacterium* tumefaciens LBA4404 carrying various antibacterial plasmids

Treatment	Total no. of explants	No. forming callus[a]	No. forming shoots (% of total) from callus[b]	
Control[c]	20	8	0	(0)
pCa2Att	20	19	4	(20)
pCa2P13	20	16	1	(5)
pCa2T4	20	13	2	(10)
pWIAtt	21	16	0	(0)

[a]After one month on selection medium Cmod containing 500 μg/ml carbenicillin and 100 μg/ml kanamycin
[b]After 4.5 months on selection medium
[c]Control was not treated with *Agrobacterium*

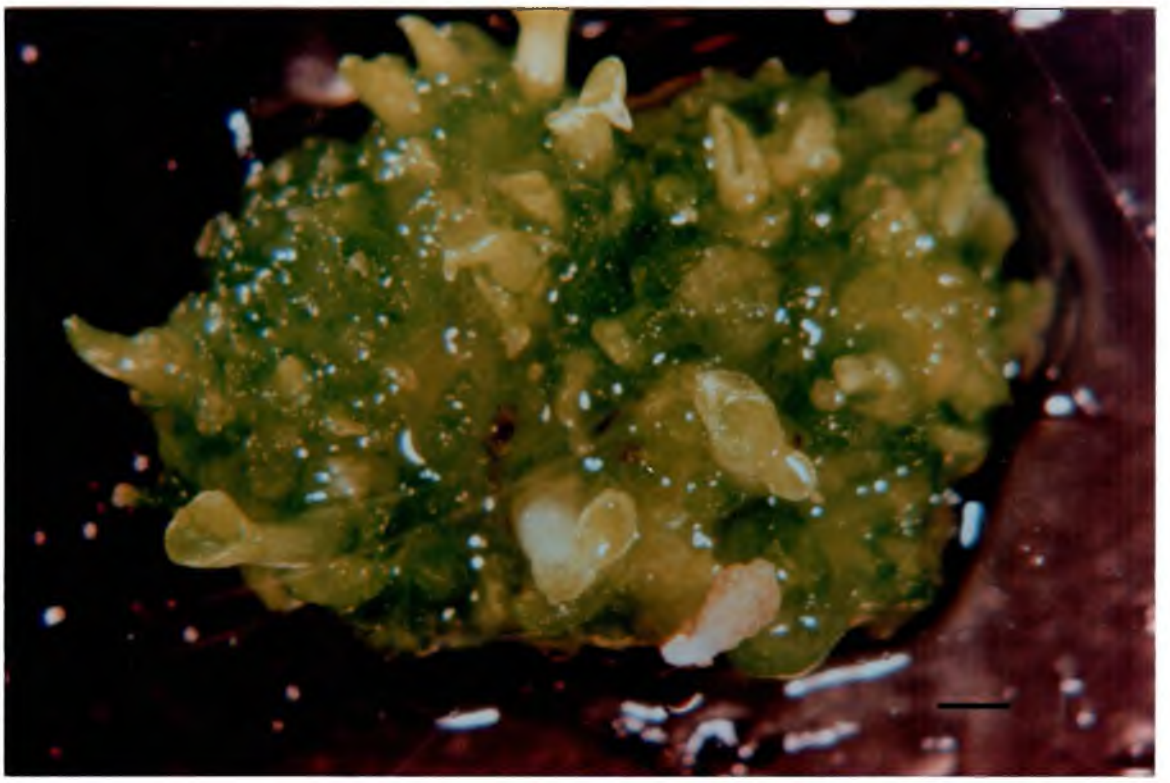

Fig. 4-4. Green shoots of UH965 regenerated from callus which originated from internodes cocultured with *Agrobacterium tumefaciens* LBA4404 carrying pCa2Att. Callus with shoots were grown on medium H1 containing 50 μg/ml kanamycin. (Bar = 1 mm)

internodes cocultivation with *Agrobacterium* carrying various plasmids ranged from 5 to 20%. No shoots were recovered from controls (Table 4-5).

In Experiment 2, UH965 plantlets were recovered following cocultivation of internodes with *Agrobacterium* carrying pCa2Att and pCa2P13. However, shoots from *Agrobacterium*/pCa2Att-treated internodes were contaminated by fungus. They were discarded without further characterization. The percentage of UH965 internodes producing kanamycin-resistant plants regenerated from this cocultivation ranged from 2.5 to 5%. No shoots were recovered from controls (Table 4-6).

Following cocultivation of half-laminae of UH965 and UH1060 in Experiment 3, callus formation was observed in all treatments including control (Table 4-7). However, many callus-bearing half-laminae turned brown and died after 3 months of culture in medium with antibiotics. Although several callus-bearing explants survived (Table 4-7) during this period, only one explant with callus later regenerated shoots. When these UH1060 shoots from cocultivation of half-laminae with *Agrobacterium* carrying pCa2P13 were cultured in H2 medium with 75 μg/ml kanamycin, most of them were kanamycin resistant. About 200 plantlets were recovered from this one explant two years after cocultivation. The percentage of kanamycin-resistant plants of UH1060 regenerated from cocultivation of half-laminae with *Agrobacterium* carrying pCa2P13 is 5%.

In the cocultivation of intact UH1060 laminae in Experiment 4, only treatment with bacteria carrying pCa2P13 produced callus. A total of three laminae formed callus. Shoots were regenerated from one of the 3 calli. The frequency of shoot regeneration following cocultivation is 3% (# forming

Table. 4-6. Response of UH965 internodes after cocultivation with *Agrobacterium tumefaciens* LBA4404 carrying various plasmids

Treatment	No. internodes cocultured	No. forming callus	No. forming shoots from callus (%)
Control[a]	40	0	0
pCa2Att	40	3	1 (2.5)
pCa2P13	40	3	2 (5)
pCa2T4	40	0	0
pWIAtt	40	0	0
pWIShiva	40	0	0

[a]Control was not treated with *Agrobacterium*

Table 4-7. Summary of cocultivation of half-lamina of UH965 and UH1060 with *Agrobacterium tumefaciens* LBA4404 carrying various antibacterial constructs

Treatment	No. expiant cocultured	No. forming callus[a]	No. forming shoot from callus[b]
UH965			
Control	20	2	0
pCa2Att	20	3	0
pCa2P13	19	1	0
pCa2T4	18	3	0
pWIAtt	17	4	0
pWIShiva	20	2	0
UH1060			
Control	20	4	0
pCa2Att	20	2	0
pCa2P13	21	14	1
pCa2T4	20	4	0
pWIAtt	20	10	0
pWIShiva	20	4	0

[a]Scored after 3.5 months. Those dead half-lamina with callus were not scored
[b]Scored after 16 months

Table 4-8. Response of UH1060 lamina cocultivated with *Agrobacterium tumefaciens* LBA4404 carrying plasmids with various antibacterial genes

Treatment	No. of lamina	No. forming callus	No. forming shoot	Percentage regeneration
pCa2Att	31	0	0	0
pCa2P13	32	3	1	3
pCa2T4	30	0	0	0
pWIAtt	30	0	0	0
pWIShiva	30	0	0	0

shoots/total laminae) (Table 4-8). Other treatments and controls did not form callus or regenerate shoots (Table 4-8).

4.3.4 Effect of nurse culture on transformation of UH965 and UH1060

In the cocultivation of UH965 internodes with or without *Agrobacterium* (Experiment 5), all treatments produced callus. The frequency ranged from 70% to 93% (Table 4-9). However, calli in the controls were much smaller than *Agrobacterium* treated ones (Fig. 4-5). With tobacco cell line 'Su' as nurse culture, the frequency of shoot regeneration after cocultivation with *Agrobacterium* was 93% in UH965 etiolated internodes, and the average number of kanamycin resistant shoots was 4.8 per explant (Table 4-9). In many explants, root also formed together with shoots (Fig. 4-6). Without nurse culture, 56% of the internodes produced shoots, and the average number of kanamycin-resistant shoots was 10.8 per explant. Controls either with or without nurse culture did not produce any shoots (Table 4-9).

In the cocultivation of UH1060 internodes in the presence of nurse culture, 32% of the internode explants produced callus. Without nurse culture, 18% of the internode explants produced callus. In controls with or without nurse culture, 2% of each treatment produced callus (Table 4-10). The calli formed in the control were smaller than pCa2Att-treated ones. Six months after cocultivation, some calli from pCa2Att-treated internodes produced multiple shoots under low light condition (Fig. 4-7). The total number of kanamycin resistant plantlets in nurse culture treatment is 319, and 366 in treatment without nurse culture (Table 4-10). The average

Table 4-9. Effect of nurse culture on the regeneration of anthurium UH965 etiolated internodes cocultivated with *Agrobacterium tumefaciens* LBA4404

Nurse[a]	Plasmid[b]	No. explant cocultured	No. forming callus[c] (%)	No. forming shoots[d] (%)	No. total Km-r[g]	No. total Km-s[g]	Average No. Km-r per total no. with shoots
yes	pCa2Att	46	43 (93)	43 (93)	206	531	4.8
no	pCa2Att	45	33 (73)	25 (56)	270	314	10.8
yes	Control[f]	45	39 (87)[e]	0 (0)	--	--	--
no	Control[f]	47	33 (70)[e]	0 (0)	--	--	--

[a]Tobacco cell line 'Su' as nurse culture
[b]Plasmid vector in *A. tumefaciens* LBA4404
[c]At least one end producing callus 6 months after cocultivation
[d]Values obtained 12 months after cocultivation
[e]Calli from controls are much smaller than from cocultured ones
[f]LB medium only
[g]Km-r, kanamycin-resistant; Km-s, kanamycin-sensitive

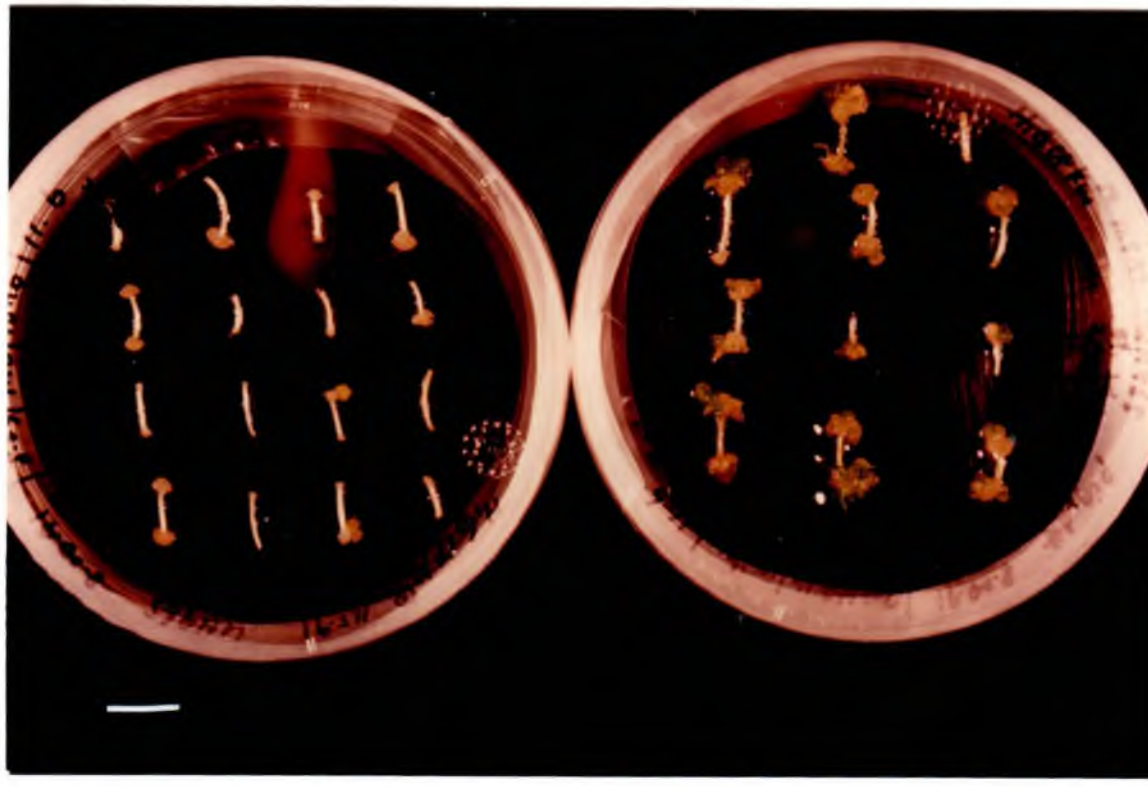

Fig. 4-5. Callus formation in cocultivated and control internodes of UH965 with tobacco Su cell line as nurse culture. Left, control; right, cocultivation with *Agrobacterium tumefaciens* LBA4404 carrying pCa2Att. Picture was taken 6 months after cocultivation. Note shoot formation in cocultured explants. (Bar = 10 mm)

Fig. 4-6. Shoot and root formation in UH965 internode cocultivated with *Agrobacterium tumefaciens* LBA4404 carrying pCa2Att. (Bar = 10 mm)

Table 4-10. Effect of nurse culture on the transformation of UH1060 internodes

Nurse	Plasmid	No. explant cocultured (# plate)	No. forming shoots (%)	Total no. of Km-s[b] shoots	Total no. of Km-r[b] shoots	Ave no. of Km-r shoots per explant
yes	pCa2Att	50 (2)	13 (26)	534	319	24
no	pCa2Att	51 (2)	16 (31)	426	366	23
yes	Control[a]	51 (2)	1 (2)	0	0	--
no	Control[a]	50 (2)	1 (2)	0	0	--

[a]LB medium only
[b]Km-r, kanamycin-resistant; Km-s, kanamycin-sensitive

Fig. 4-7. Multiple shoot formation in UH1060 internodal callus after cocultivation with *Agrobacterium tumefaciens* LBA4404 carrying pCa2Att. (Bar = 10 mm)

number of kanamycin-resistant shoots in nurse culture treatment is 24 per internode, and in treatment without nurse culture is 23 per internode (Table 4-10). These shoots were subsequently transferred to H2 medium with 75 μg/ml kanamycin, and cultured under higher light to allow root formation and to select for kanamycin- resistant plantlets.

4.3.5 De novo callus induction from lamina culture of renegerated kanamycin-resistant anthurium

When lamina explants of kanamycin-resistant UH965 plantlets were cultured on C medium with 50 μg/ml kanamycin, 50 to 60% formed callus in five months. One of the plates, designated 1-4-3, is shown in Fig. 4-8. These calli can serve as source material for isolation of nucleic acids and proteins. Among 147 lamina explants, one had bacteria outgrowth.

4.3.6 Root explants as a source for cocultivation with *Agrobacterium*

The observation of regeneration of shoots from root explants (Chapter 3) lead to a hypothesis that root explants could be used for cocultivation with *A. tumefaciens*. The result of this experiment is shown in Table 4-11. Only a few root explants cocultivated with *Agrobacterium* formed callus under selection conditions. Almost no callus was observed in control explants except one in UH1060, which even formed shoots under low kanamycin selection (25 μg/ml). In the first two months after cocultivation, no callus was observed in any treatments in H1 medium containing cefotaxime and 50 μg/ml kanamycin, thus the kanamycin concentration was reduced to

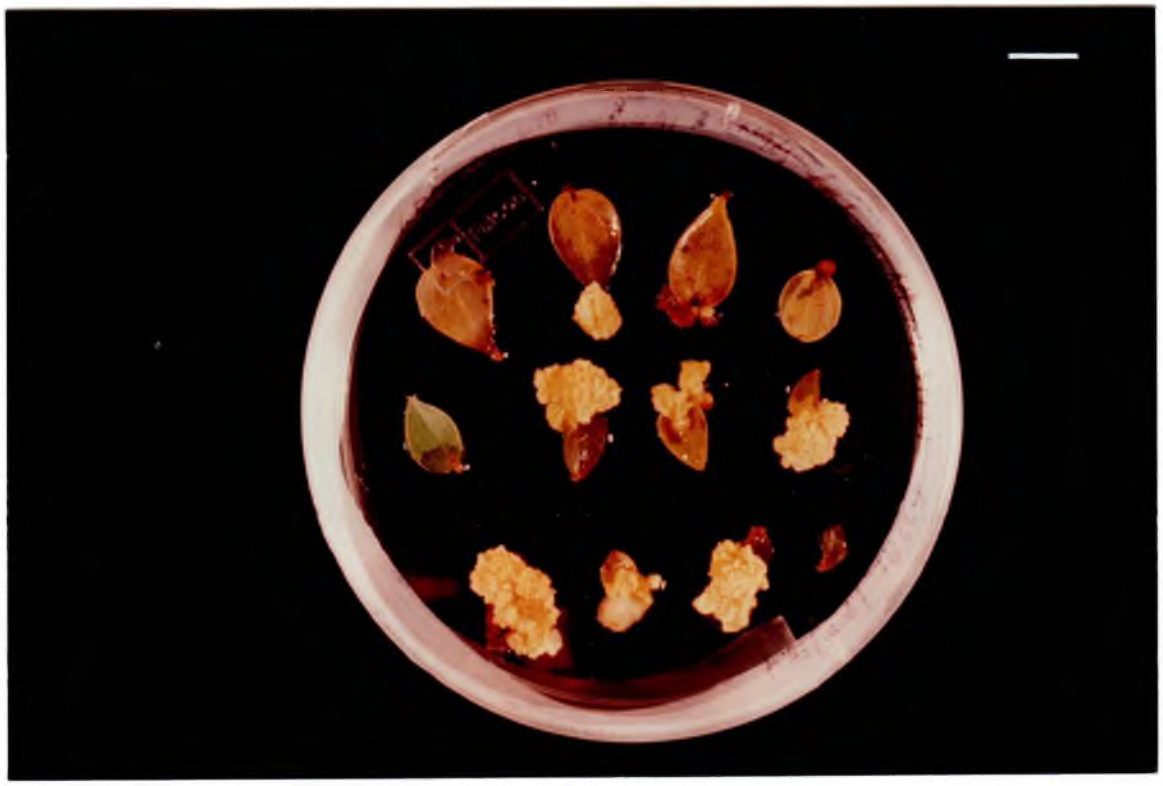

Fig. 4-8. De novo callus formation in lamina culture of kanamycin-resistant UH965 regenerated from etiolated internodes previously cocultivated with *Agrobacterium tumefaciens* LBA4404 carrying pCa2Att. Calli were induced and subcultured on C medium containing 50 μg/ml kanamycin in the dark. (Bar = 10 mm)

Table 4-11. Summary of cocultivation of root explants of anthurium with *Agrobacterium tumefaciens* LBA4404 carrying pCa2Att

Cultivar	Treatment	No. root explants (# plate)		No. forming yellow callus	No. green callus
UH965	pCa2Att	53	(2)	3	1
	Control[a]	17	(1)	0	0
UH1003	pCa2Att	28	(1)	2	0
	Control[a]	15	(1)	0	0
UH1060	pCa2Att	26	(1)	0	0
	Control[a]	21	(1)	2	2[b]
Anuenue	pCa2Att	77	(2)	2	1[b]
	Control[a]	25	(1)	0	0
Mauna Kea	pCa2Att	48	(2)	0	0
	Control[a]	25	(1)	0	0

[a]LB medium only
[b]Regenerated multiple shoots

25 μg/ml in the next transfer. Under this selection, some calli of UH965 (3 pieces), UH1003 (2 pieces), UH1060 (2 pieces in control), and 'Anuenue' (2 pieces), formed after 7 months of culture. After 11 months, only one callus from 'Anuenue' regenerated shoots. In the UH1060 control, two calli also regenerated shoots (Table 4-11). These shoots were transferred to H2 medium containing 75 μg/ml kanamycin under higher light condition (32 μE $m^{-2}sec^{-1}$, 16 hours light/8 hours dark cycle) to allow root formation and to select for kanamycin-resistant plantlets.

4.3.7 GUS activity in organs of kanamycin-resistant anthurium

From kanamycin-resistant UH965 and UH1060 plantlets, a total of 28 different leaf segments and 28 different root segments were used for GUS staining. No positive GUS reaction (blue spot) in leaf segments or roots was observed using histochemical staining at 37^{o}C for 3 days (Table 4-12).

4.3.8 Amplification of introduced genes

PCR-amplified DNA fragments from the first coculture-derived UH965 and UH1060 plantlets are shown in Fig. 4-9 and Fig. 4-10. The amplified NPTII gene fragment (Fig. 4-9) of kanamycin-resistant UH965 plantlets transformed with pCa2Att (Fig. 4-9, lanes 2-5) and kanamycin-resistant UH1060 plantlets transformed with pCa2Att (Fig. 4-9, lanes 6-7) showed the expected size of 1054 bp. An NPTII fragment of similar mobility was amplified from the plasmid pBI121 (Fig. 4-9, lane 8). No amplification was observed in untransformed control DNA (UH965) (Fig. 4-9, lane 1). The

Table 4-12. Summary of ß-glucuronidase (GUS) histochemical staining in kanamycin-resistant anthurium tissues

Cultivar	Tissue	No. stained	No. blue spots
UH965 Ca2Att	Leaf	11	0
	Root	10	0
UH1060 Ca2Att	Leaf	8	0
	Root	7	0
Ca2P13	Leaf	9	0
	Root	11	0

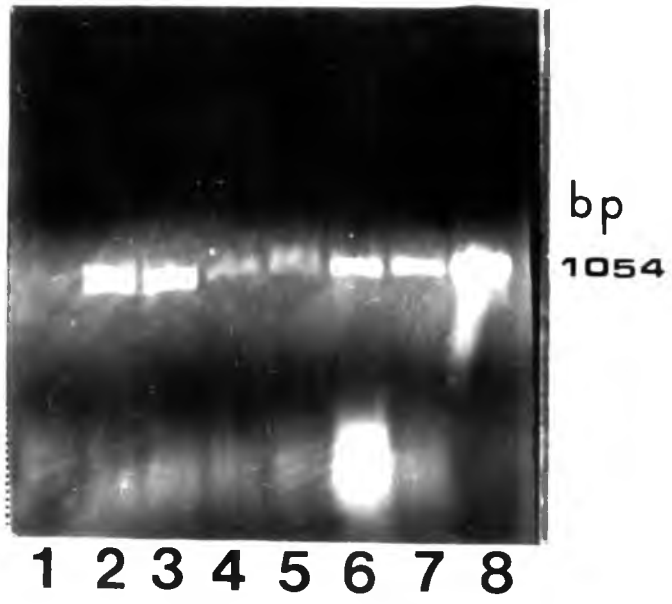

Fig. 4-9. PCR analysis of DNA from kanamycin-resistant anthurium plantlets of cultivars UH965 and UH1060. DNA isolated from leaf or callus tissue served as template for PCR amplification using primers specific for the NPTII gene. These primers amplified a 1054 bp fragment as indicated. Lanes: 1, Untransformed UH965 DNA (negative control); 2, DNA from UH965 Ca2Att #1-1; 3, DNA from UH965 Ca2Att #1-2; 4, DNA from UH965 Ca2Att #1-3; 5, DNA from UH965 Ca2Att #1-4; 6, DNA from UH1060 Ca2Att #2 callus; 7, DNA from UH1060 Ca2Att #4 callus; 8, pBI121 plasmid (positive control).

Fig. 4-10. PCR amplification of the attacin gene from the DNA of kanamycin-resistant plantlets and callus. The two primer sites located within the *Att* gene should amplify a fragment of 546 base pairs. Lanes: 1, Untransformed UH965 leaf DNA; 2, UH965 Ca2Att #1-1 leaf DNA; 3, UH965 Ca2Att #1-2 leaf DNA; 4, UH965 Ca2Att #1-3 leaf DNA; 5, UH965 Ca2Att #1-4 leaf DNA; 6, UH1060 Ca2Att #2 callus DNA; 7, pCa2Att plasmid. The molecular weight markers lambda phage *Hin*d III digests are in lane 8.

amplified *Att* gene fragment of 546 bp from kanamycin-resistant anthurium plantlets (Fig. 4-10, lanes 2-5, UH965-Ca2Att-1-1 to 1-4; lane 6, UH1060-Ca2Att #4) had the same mobility as the *Att* fragment from the plasmid pCa2Att (Fig. 4-10, lane 7). The untransformed control DNA did not show fragment amplification (Fig. 4-10, lane 1).

PCR amplification of DNA from plantlets recovered following cocultivation of UH965 internodes with and without nurse cultures (Experiment 5) is shown in Fig. 4-11. DNA samples from kanamycin-resistant UH965 plantlets were amplified for *Att*, *nptII* and *gus* genes, respectively (Fig. 4-11, lanes 2-6). The three gene sequemces were amplified in the expected sizes and confirmed by amplified fragments of plasmid pCa2Att (Fig. 4-11, lane 7). No *gus* amplification was observed for the DNA from the second sample (data not shown).

Pooled DNA from two kanamycin-resistant UH1060 plantlets of a single clone, regenerated following cocultivation of internodes with *Agrobacterium* carrying pCa2Att without tobacco cell 'Su' as nurse (Experiment 4), were amplified for *Att*, *nptII* and *gus* genes, respectively (Fig. 4-12). Amplified *Att* (Fig. 4-12, lane 2) and *nptII* (Fig. 4-12, lane 3) gene fragments of UH1060 DNA have the same mobility as those amplified from plasmid pCa2Att (Fig. 4-12, lane 5, 6,). The gus gene fragment was not amplified from UH1060 DNA (Fig. 4-12, lane 4).

The amplified DNA from shoots and callus derived from UH1060 detached laminae (Experiment 4) transformed with pCa2P13 is shown in Fig. 4-13. In this set of amplifications, the 3' primer of nptII was smaller (Table 4-3) so that the size of amplified *nptII* gene fragment would be 1048 bp. The size of amplified *P13* and *nptII* genes from two samples

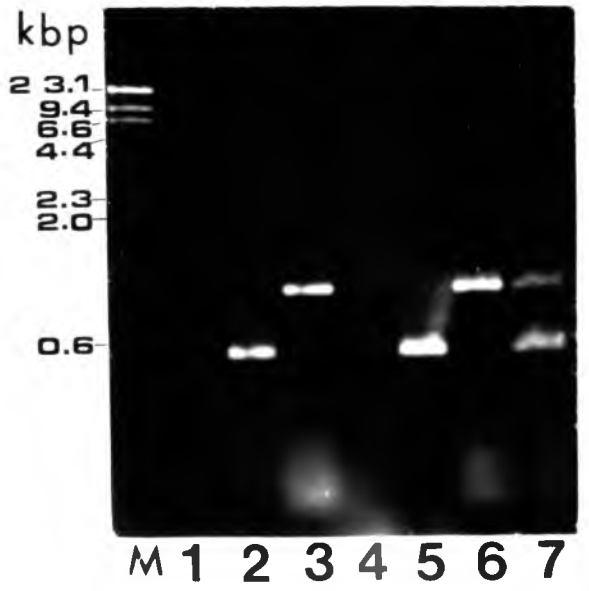

Fig. 4-11. PCR analysis of the DNA from kanamycin-resistant UH965 anthurium plantlets. DNA isolated from leaf tissue served as template for PCR amplification using primers for *Att*, *nptII* and *gus* genes. The primers should amplify 3 fragments of 546 base pairs (bp), (*Att* gene), 1054 bp (*nptII* gene), and 1724 bp (*gus* gene). Lanes: 1, Untransformed UH965 DNA; 2-4, UH965 Ca2Att-N 1-17 DNA amplified with primers for *Att*, *nptII* and *gus*, respectively; 5-6, UH965 Ca2Att-NN 1-15 DNA amplified for *Att* and *nptII*, respectively; 7, pCa2Att plasmid DNA amplified for *Att*, *nptII* and *gus* in the same tube. M, lambda *Hind* III digests as molecular weight markers. N, from cocultivation with 'Su' nurse culture; NN, from cocultivation without nurse culture.

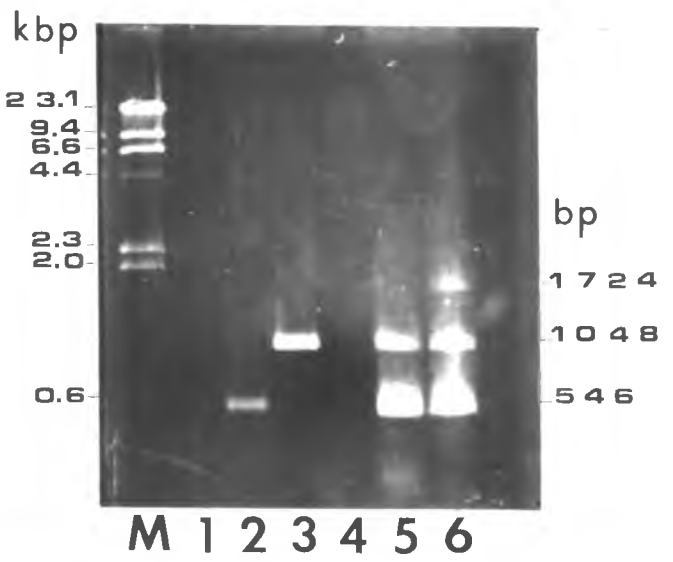

Fig. 4-12. PCR amplification of the pooled DNA from kanamycin-resistant UH1060 anthurium plantlets regenerated following cocultivation of etiolated internodes with *Agrobacterium tumefaciens* LBA4404 carrying pCa2Att without tobacco cell 'Su' nurse culture. DNA isolated from leaf tissue served as template for PCR amplification using primers for *Att*, *nptII* and *gus*. The primers should amplify 3 fragments of 546 base pairs (bp) (*Att*), 1048 bp (*nptII*), and 1724 bp (*gus*). Lanes: 1, Untransformed control UH965 DNA; 2-4, UH1060 Ca2Att-NN #12 DNA amplified with primers for *Att*, *nptII* and *gus*, respectively; 5, *Att*, *nptII* and *gus* gene fragments amplified from 10-fold diluted DNA samples of lane 6; 6, pCa2Att amplified for the same three gene fragments. M, lambda DNA digests as molecular markers.

(Fig. 4-13, lanes 3-6) were as expected (313 bp and 1048 bp, respectively) and were similar to the pCa2P13 plasmid amplified genes (Fig. 4-13, lane 7). The *gus* gene was amplified in one of the sample (UH1060 Ca2P13 #1), to the expected size of 1724 bp, but with less intense band on agarose gel (data not shown). Its has the same mobility as amplified *gus* gene fragment of pCa2P13 DNA. No *gus* amplification was observed from the second sample.

4.3.9 Southern blots

Genomic DNA isolated from in vitro grown plants of kanamycin-resistant UH1060 anthurium (Ca2P13 plants from Experiment 4) were resistant to restriction enzyme digestion (*Eco* RI, *Hin*d III, and *Sma* I). After agarose gel electrophoresis and ethidium bromide staining, high molecular DNA, larger than 23 kbp as compared to *Hin*d III digested lambda DNA molecular weight markers, were still visible on the gel. After Southern blot hybridization with a nonradioactive P13 gene probe, only high molecular DNA hybridized to the *P13* probe (Fig. 4-14).

Southern blot hybridizations of PCR-amplified *nptII* and *Att* genes from DNA of putatively transformed UH965 and UH1060 plantlets (Experiment 5) using digoxigenin-11-dUTP labeled *nptII* and *Att* probes are shown in Fig. 4-15 and Fig. 4-16. Fragments amplified to the expected size of 1054 bp and 546 bp hybridized to specific probes for *nptII* and *Att*, respectively. No hybridization was observed in untransformed controls.

Att gene amplified from DNA of putatively transformed UH965 plantlets (Fig. 4-11, lanes 2 and 5) also hybridized to the nonradioactive

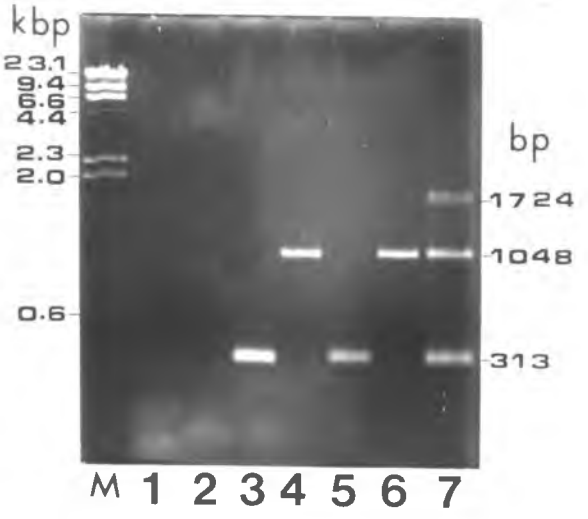

Fig. 4-13. PCR analysis of the DNA from UH1060 shoot and calli. DNA was amplified with primers specific for the *P13*, *nptII* and *gus* genes corresponding to fragment sizes of 313 base pairs (bp)(*P13*), 1048 bp (*nptII*), and 1724 bp (*gus*). The 3' downstream primer of *nptII* in this amplification produces a fragment shorter than in Fig. 4-9A. Lanes: 1, Amplification without DNA; 2, Untransformed UH1060 leaf DNA; 3, UH1060 Ca2P13 #1 callus with shoot DNA amplified for *P13*; 4, same as lane 3, but amplified for *nptII*; 5, UH1060 Ca2P13 #2 callus with shoot DNA amplified for *P13*; 6, same as lane 5, but amplified for *nptII*; 7, pCa2P13 plasmid amplified for *P13*, *nptII* and *gus* in the same tube. M, lambda *Hin*d III-digested molecular weight markers.

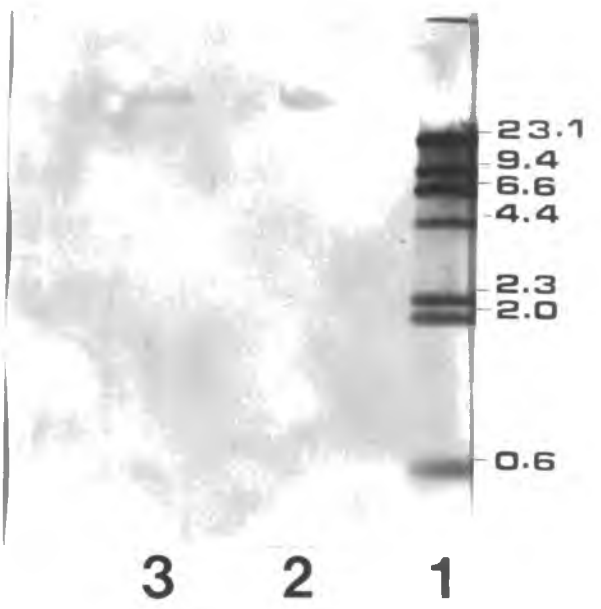

Fig. 4-14. Southern blot hybridization of genomic DNA of kanamycin-resistant UH1060 plants regenerated following cocultivation of lamina with *Agrobacterium tumefaciens* LBA4404 carrying pCa2P13. DNA (10 ug) were digested with *Hind* III overnight, and separated in 0.6% agarose. The DNAs were transferred onto Zetabind nylon membrane and hybridized with nonradioactive *P13* probe. Lanes: 1, lambda *Hind* III digest as molecular markers; 2, UH1060 Ca2P13 #1 DNA; 3, UH1060 Ca2P13 #2 DNA. Only high molecular weight DNA hybridized with the probe due to incomplete restriction digestion.

Fig. 4-15. Southern blot of PCR-amplified *nptII* gene from UH965 DNA. DNA blot was hybridized with digoxigenin-11-dUTP labeled *nptII* gene probe. The samples are the same as described in Fig. 4-9.

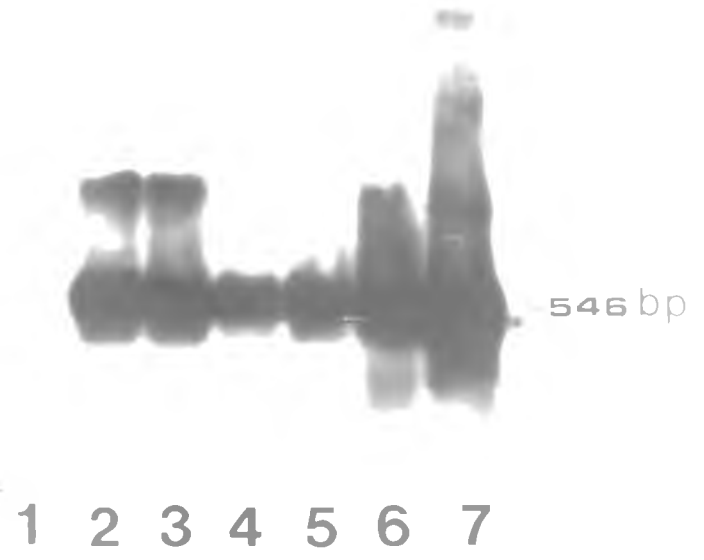

Fig. 4-16. Southern blot analysis of PCR-amplified *Att* gene from DNA of UH965 kanamycin-resistant plantlets. DNA blot was hybridized to digoxigenin-11-dUTP labeled *Att* gene probe. The samples are the same as described in Fig. 4-10.

Att probe at 546 bp (Fig. 4-17). No attempt was made to hybridize the *nptII* band (Fig. 4-17, lanes 3, 6, and 7), since they all had the expected size of 1054 bp. *Att* gene amplified from pooled DNA of putatively transformed UH1060 plantlets (from Experiment 5) hybridized to the nonradioactive *Att* probe (Fig. 4-18, lane 2). An attempt was made to strip off the *Att* probe from the same DNA blot according manufacturer's directions (Boehringer Mannheim). However, after hybridization to the second probe, *nptII* probe, hybridization in both *Att* and *nptII* bands still showed in the same X-ray film (Fig. 4-18). Therefore the protocol of stripping the probe off the membrane has to be modified. The *nptII* gene fragment amplified from kanamycin-resistant anthurium DNA hybridized with the nonradioactive *nptII* probe (Fig. 4-18, lane 3). The positive controls (amplified from pCa2Att) have the same hybridization pattern as *Att* and *nptII* fragments amplified from UH1060 DNA (Fig. 4-18, lanes 5, 6,).

A membrane containing the *P13* and *nptII* gene fragments amplified from DNA of putatively transformed UH1060 plantlets (from Experiment 4) was hybridized to a nonradioactive *P13* probe. The result is shown in Fig. 4-19. The probe hybridized to a major band visible in the ethidium bromide stained agarose gel (Figs. 4-13, 4-19, lanes 3, 5, and 7). A minor band with higher molecular weight was also observed on Southern blot in these samples (Fig. 4-20). The *P13* probe made by PCR amplification incorporating digoxigenin-11-dUTP also resulted with two visible bands on the non-denaturing gel (Fig. 4-20). The reason for this phenomenon is not clear. The *nptII* amplified from the same UH1060 DNA was not tested for hybridization with *nptII* probe, since it has the same expected size as previously shown in another Southern blot (Fig. 4-15).

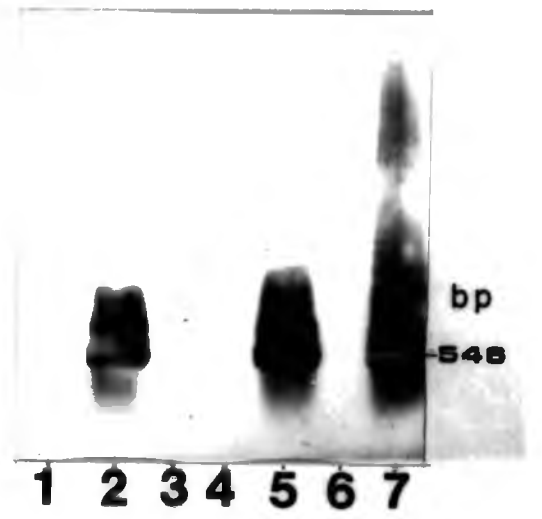

Fig. 4-17. Southern blot hybridization of PCR-amplified *Att* gene from UH965 DNA. The samples are the same as described in Fig. 4-11.

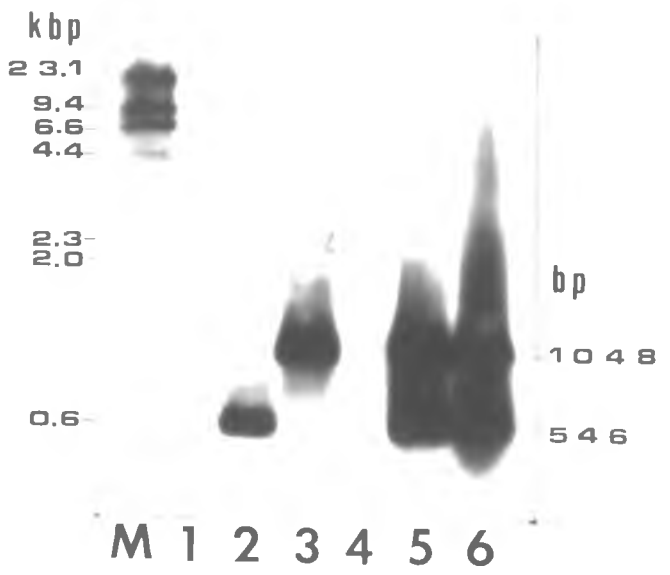

Fig. 4-18. Southern blot hybridization of PCR-amplified UH1060 *Att* and *nptII* gene fragments. The samples are the same as described in Fig. 4-12. The hybridized DNA blot was reacted with Lumi-Phos 530 for 30 minutes at 37^{o}C and exposed to X-ray film by chemiluminescence for 3 to 5 minutes at 25^{o}C. This blot is a combination from two separate hybridizations with *Att* and *nptII* probes.

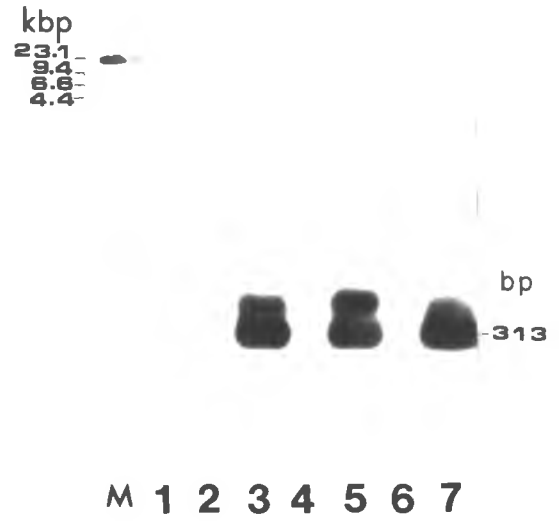

Fig. 4-19. Southern blot hybridization of amplified UH1060 *P13* gene. DNA blot was probed with digoxigenin-11-dUTP labeled *P13* probe. The samples are the same as described in Fig. 4-13.

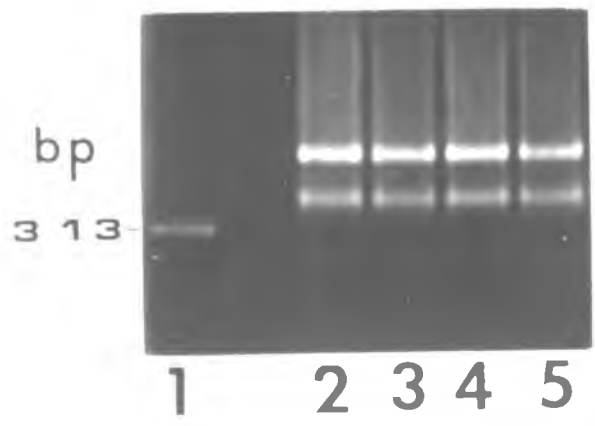

Fig. 4-20. Preparation of nonradioactive *P13* probe by PCR amplification of pCa2P13 plasmid. Lanes: 1, *P13* fragment amplified with four deoxyribonucleotides (dNTPs); 2-5, *P13* fragments amplified from lane 1 sample with dNPTs plus digoxigenin-11-dUTP. DNA samples were separated in 0.8% agarose.

4.3.10 Western blots

De novo calli induced from lamina cultures of kanamycin-resistant anthurium were assayed for expression of the attacin protein. A polyclonal antibody raised against partially purified attacin from the cecropia insect cross-reacted with a band of approximately 25 kDa in kanamycin-resistant calli (Fig. 4-21, lanes 2-4). This band had the same mobility in the denaturing gel as the partially purified attacin (Fig. 4-21, lane 5). No cross-reaction for this specific band was detected in untransformed plant tissue (callus) (Fig. 4-21, lane 1). The same 25 kDa protein band was also detected in three other protein samples from transformed plants. Proteins of sizes other than 25 kDa (15 kDa and several proteins larger than 31 kDa) were also recognized by the antibody in both untransformed and kanamycin-resistant anthurium tissues (Fig. 4-21), and were not considered to be due to expression of the introduced antibacterial gene *Att*.

A polyclonal antibody against T4 lysozyme was used to detect T4 *e* gene expression in leaf tissue of kanamycin-resistant anthuriums. However, the antibody cross-reacted to many bands. Even though induced T4 lysozyme was visible in a denaturing gel in a time-course study of induction (Fig. 4-22, arrow), the antibody cross-reacted to almost every band, therefore no conclusion could be made with regard to T4 lysozyme cross-reactivity. In a separate assay, the antibody was cross-reacted to total bacterial (DH5α) extract at 37^{o}C overnight without introduced T4 *e* gene to reduce the background proteins before detection. No specific binding to the induced T4 lysozyme or protein extracts of anthurium could be detected.

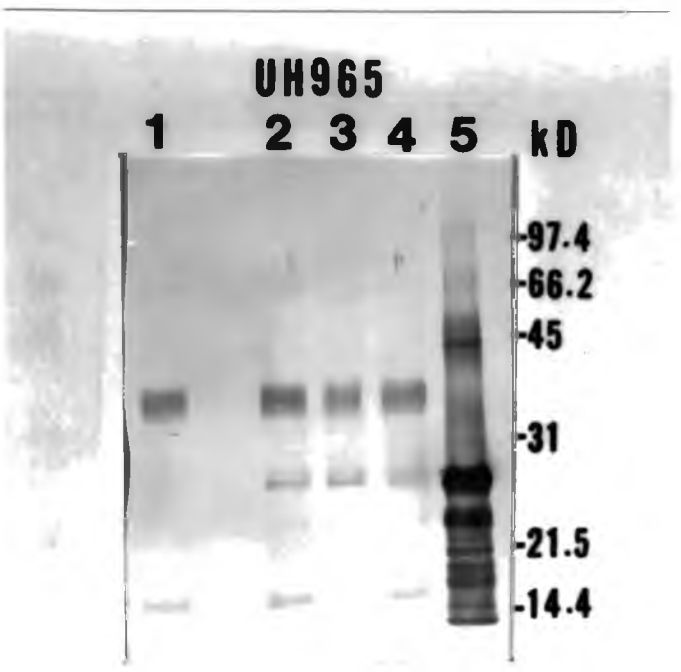

Fig. 4-21. Western blot of UH965 Ca2Att plants. Protein extracts from de novo callus on lamina of kanamycin-resistant plants were separated in 12% SDS-PAGE. The proteins were electroblotted onto a nitrocellulose membrane and reacted with polyclonal antibody against attacin. Lanes: 1, Untransformed UH965 callus; 2-4, transformed UH965 lamina callus; 5, partially purified cecropia insect attacin.

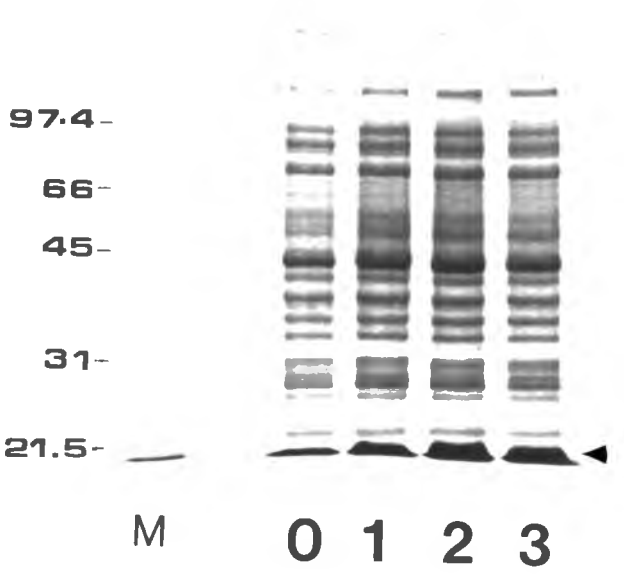

Fig. 4-22. SDS-PAGE analysis of induced T4 lysozyme (arrow) in *E. coli* DH5α cells transformed with pHSe5. The total protein were denatured and separated in 10% SDS-polyacrylamide gel, and stained with Coomassie blue R-250 dye. Lanes: M, molecular weight marker; 0, Zero time after addition of IPTG; 1, one hour after induction; 2, 2 hours after induction; 3, 3 hours after induction.

Therefore, the expression of T4 lysozyme in kanamycin-resistant UH965 anthurium could not be assessed during this time.

4.3.11 Resistance to *X. campestris* pv. *dieffenbachiae* D150 in kanamycin-resistant anthurium

In the first challenge experiment, the inoculated UH965 plants were incubated in the air-conditioned laboratory (23-25^{o}C) under grow-lights for three weeks. During this time, no symptom development was observed in untransformed or kanamycin-resistant plantlets. The inoculated plantlets were then transferred to a growth chamber at 28^{o}C with relative humidity 71%. After three weeks, blight symptoms developed in most of the control plants. Eight out of nine control plants had leaf chlorosis, and seven had leaf necrosis (Table 4-13). Seven out of nine control plants had stem and root rot. From the control plants except one (plant #9) *Xcd*150 colonies (more than 150 cfu/20 μl extract) were reisolated from the tissues. Control plant #9 showed no disease symptom (Table 4-13).

In kanamycin-resistant plants carrying Ca2Att, six plants were symptomless (plants #2, 3, 6, 9, 12, 13, Table 4-13). Only three plants showed either mild leaf chlorosis or necrosis with little or no water-soaking (plants # 7, 8 and 10, Table 4-13). However, 5 out of 13 plants showed water-soaked spots on the leaves. When *Xcd*150 was reisolated from the inoculated petioles and stem base, 11 plants (plant #1 to 8, and 10 to 12) had more than 150 cfu per 20 μl of extract in at least one of the petiole sections, including symptomless plants. Six other plants (#2, 3, 4, 10, 11,

Table 4-13. Symptom development and reisolation of *Xanthomonas campestris* pv. *dieffenbachiae* strain D150 from challenged kanamycin-resistant and untransformed UH965 anthuriums

Plant No.	Symptom				Reisolation of bacteria (cfu)[a]			
	None	Chl	Nec	Stem rot WS	None	1-100	101-150	>150
Untransformed								
1		x	x	x				x
2		x	x	x				x
3		x	x	x				x
4		x	x	x				x
5		x		x				x
6		x	x					x
7		x	x	x				x
8		x	x	x				x
9	x				x			
Transformed with pCa2Att								
1				x				x
2	x						x	x
3	x					x		x
4				x		x		x
5				x				x
6	x							x
7		x						x
8			x	x				x
9	x				x			
10			x			x		x
11				x		x		x
12	x						x	x
13	x				x			

chl, leaf chlorosis; Nec, leaf necrosis; WS, water soaked spots; cfu, colony forming unit per 20 ul extract in ET medium

[a]Bacterial reisolation from petiole and stem sections

and 12) had fewer than 150 cfu, and two (#9 and 13) had no bacteria from any petiole sections (Table 4-13).

A noticeable difference among untransformed controls and kanamycin-resistant plants is that all kanamycin-resistant plants had intact stem and root systems, as compared to only 2 plants in control. The appearance of the challenged control and transformed plants is shown in Fig. 4-23.

In the second challenge experiment, one pot each of putative transformed Ca2Att, Ca2P13, and Ca2T4 plants were evaluated. In untransformed controls, 9 out of 13 plants had leaf chlorosis (plants #1, 2, 4,6, 7, 8, 9, 11, and 12, Table 4-14), and 5 among these plants had leaf necrosis (plants #1, 2, 4, 7, and 9, Table 4-14). In addition, whole plant wilting was observed in two control plants (# 7 and 11). Four out of these 9 plants had stem rot (#2, 4, 5, and 7). One of these had stem and root rot and the whole plant was dessicated (#5). Four plants had water-soaked spots (#6, 8, 9, and 11). All inoculated petioles showed either total or partial necrosis, and one plant was decomposed due to stem rot. Three out of 13 tested control plants showed no symptoms (#3, 10, and 13). For bacteria reisolation, all control plants had more than 150 cfu per 20 μl extract in some or all petiole sections, including symptomless plants. Five plants (# 3, 4, 10, 11, and 13) had between 0 and 100 cfu/20 μl of reisolated *Xcd*150 in one of the petiole sections (Table 4-14).

In kanamycin-resistant UH965 Ca2Att plants, there were basically no symptoms, except that four of 8 tested plants (#1, 2, 6, and 7) had partial necrosis on the inoculated petiole (Fig. 4-24A, Table 4-14). Seven plants (#1, 2, 3, 4, 6, 7, and 8) had 0-100 cfu/20 μl of *Xcd*150, and usually no

Fig. 4-23. Challenge of kanamycin-resistant and untransformed UH965 anthurium plants with blight pathogen *Xanthomonas campestris* pv. *dieffenbachiae* strain D150. The plants were incubated at 22-25^{o}C for three weeks at low humidity, then at 28^{o}C in a humidified growth chamber for three weeks. The picture was taken at 7th week after challenge (or 4th week after incubation at 28^{o}C). Left, control; right, kanamycin-resistant Ca2Att anthurium plants. (Bar = 17 mm)

Table 4-14. Symptom development and reisolation of *Xanthomonas campestris* pv. *dieffenbachiae* strain D150 from of challenged kanamycin-resistant and untransformed UH965 anthurium

Plant No.	Symptom							Reisolation of bacteria (cfu)[d]			
	None	Chl	Nec	Sr	Rr	WS	PN[c]	None	1-100	101-150	>150
Untransformed											
1		x	x				2				x
2		x	x	x			2				x
3	x						0		x		x
4		x	x	x			2		x		x
5				x	x[a]		3				x
6		x				x	1				x
7		x[b]	x	x			2				x
8		x				x	1				x
9		x	x			x	2				x
10	x						0		x		x
11		x[b]				x	2		x		x
12		x					1				x
13	x						0		x		x
Transformed with pCa2Att											
1							1		x		x (tip)
2							1		x		x
3	x						0		x		x
4	x						0		x		x (tip)
5	x						0				x
6							1		x		x
7							1		x		x
8	x						0		x		
Transformed with pCa2P13											
1						x	0				x
2	x						0	x			
3						x	1		x		x
4							0		x(middle)		x
5	x						0	x			
6	x						0	x			
7	x						0				x
8	x						0		x (base)		x
9		x		x			2		x		x (base)

Chl, leaf chlorosis; Nec, leaf necrosis; Sr, stem rot; Rr, root rot; WS, water soaked spots; cfu, colony forming unit per 20 ul extract spotted on ET medium

[a]Whole plant rot and dry

[b]whole plant wilting

[c]Petiole necrosis: 3, whole petiole severely necrotic; 2, whole petiole necrotic; 1, part of the petiole necrotic; 0, no necrosis

[d]Bacterial reisolation from petiole and stem sections

Table 4-14. (continued) Symptom development and reisolation of *Xanthomonas campestris* pv. *dieffenbachiae* strain D150 from of challenged kanamycin-resistant and untransformed UH965 anthurium

Plant No.	Symptom							Reisolation of bacteria (cfu)[d]			
	None	Chl	Nec	Sr	Rr	WS	PN[c]	None	1-100	101-150	>150
Transformed with pCa2T4											
1							1				x
2							2				x
3	x						0				x
4							2		x (middle)		x
5							1				x
6							2		x (middle)		x
7							2		x		
8	x						0	x			
9		x[b]					1		x		x
10							1				x
11							1				x

Chl, leaf chlorosis; Nec, leaf necrosis; WS, water soaked spots; cfu, colony forming unit per 20 ul extract on ET medium

[a]Whole plant rot and dry

[b]whole plant wilting

[c]Necrosis: 3, whole petiole severely necrotic; 2, whole petiole necrotic; 1, part of the petiole necrotic; 0, no necrosis

[d]Bacterial reisolation from petiole and stem sections

detectable *Xcd*150 were recovered from several of the petiole sections. Seven of the plants (#1 to #7) had more than 150 cfu/20 μl extract in only one part of the petiole sections (Table 4-14).

In putatively transformed UH965 with pCa2P13, 5 out of 9 tested plants (plants #2, 5, 6, 7, and 8) had no symptoms (Fig. 4-24B, Table 4-14). Plant #1 and #3 both had water-soaked spots on the leaf, and the petiole of plant #3 had mild necrosis. Plant #9 had mild leaf chlorosis and stem rot, and necrosis on its inoculated petiole. No *Xcd*150 colonies were recovered from the petiole extracts of three out of the 5 symptomless plants (#2, 5, and 6). Four of the 9 plants (#3, 4, 8, and 9) had fewer than 100 cfu/20 μl extract in various parts of the inoculated petioles. Six plants (#1, 3, 4, 7, 8, and 9) had more than 150 cfu/20 μl extract of *Xcd*150 in other parts of the petioles.

In UH965 plants putatively transformed with pCa2T4, 2 out of 11 tested plants (plant #3 and 8) had no symptoms; all other plants showed either total or partial petiole necrosis (Fig. 4-24C, Table 4-14). Plant #9 also was wilted. Plant #8 had no detectable *Xcd*150 bacteria in the petiole extract. Plant #1 to #6 and #9 to #11 all had more than 150 cfu/20 μl extract in certain sections or all sections of the petiole. Plant #4 averaged 17 cfu/20 μl extract in the middle section of the petiole. Two other sections had bacteria more than 150 cfu/20 μl extract. Plant #7 only had bacteria in the middle section of the petiole (average 2.4 cfu/20 μl); two other sections had no detectable bacteria. Plant #9 had fewer than 100 cfu/20 μl of the *Xcd*150 in top and middle sections of the petiole (Table 4-14).

Fig. 4-24A. Challenge of anthurium with blight bacteria *Xanthomonas campestris* pv. *dieffenbachiae* D150. Left, untransformed control; right, kanamycin-resistant UH965 Ca2Att plants. (Bar = 20 mm)

Fig. 4-24B. Challenge of anthurium with blight bacteria *Xanthomonas campestris* pv. *dieffenbachiae* D150. Left, untransformed UH965 control; right, kanamycin-resistant UH965 Ca2P13 plants. (Bar = 20 mm)

Fig. 4-24C. Challenge of anthurium with blight bacteria *Xanthomonas campestris* pv. *dieffenbachiae* strain D150. Left, untransformed UH965; right, UH965 putatively transformed with pCa2T4. (Bar = 22 mm)

The challenged plants were photographed before bacterial reisolation. The contrast is apparent between the control and pCa2Att (Fig. 4-24A), pCa2P13 (Fig. 4-24B), and pCa2T4 (Fig. 4-24C). Most of the treated plants showed vigorous growth, while most of the control plants showed weaker growth or wilting. The results from both the appearance of the tested plants and the bacterial reisolation suggest that putatively transformed plants carrying antibacterial genes had partial or total resistance to one strain (*Xcd*150) of the blight pathogen.

4.4 DISCUSSION

The bactericidal or bacteriostatic activity of four tested proteins in microdilution assay were observed under several concentrations. Interestingly, the antibacterial protein Shiva10 at low concentration, 1.25 μg/ml, was inhibitory to bacterial growth. Cecropin B was previously found to be active against Gram-negative bacteria (Boman et al., 1991). Both SB37 and Shiva10 are derivatives of the cecropin B. Introduction of the corresponding cecropin B, its derivatives, SB37 and Shiva10 genes into anthurium may have potential against blight bacteria. When the attacin E was added to *E. coli* D31 cells in rich medium, and the number of surviving bacteria estimated after different times by spreading samples on agar plates, attacin E was found to reduce the viable bacterial colonies up to 10^4-folds (Hultmark et al., 1983).

Bactericidal activity of cecropins was observed by Hultmark et al. (1982) using inhibition zone assays in the wells of agar plates. No inhibition activity of cecropin B, SB37 and attacin was observed in the filter disc study. The

reason could be due to the absorption of the proteins by filter paper discs rather than diffusion of the proteins into the agar medium.

Agrobacterium tumefaciens LBA4404 carrying a gene for one of the four antibacterial proteins (attacin) in the expression vector pBI121 was used (due to its availability then) in cocultivation experiments. Other antibacterial proteins were very promising for potential control of pathogenic bacteria based on the results of the bactericidal assay. Introduction of the gene constructs for the other 3 proteins into anthurium might confer bacterial disease resistance.

An optimal kanamycin concentration is necessary to allow transformed cells to grow while inhibiting the growth of untransformed cells. The formation of callus was completely inhibited by 75 μg/ml kanamycin during UH965 internode culture and by 50 μg/ml during lamina culture. Callusing on both internode and lamina cultures of UH1060 was inhibited by 50 μg/ml kanamycin. Based on this result, a kanamycin concentration of 50 μg/ml was chosen for culture of anthurium explants treated with *Agrobacterium*. Regenerated plantlets were cultured in media containing 50 μg/ml, then moved onto media with 75 μg/ml to select for putative kanamycin resistance. The level of kanamycin concentration that completely inhibits plant growth was not determined.

Cocultivation of internodes and laminae with *Agrobacterium tumefaciens* LBA4404 carrying various plasmids, pCa2Att and pCa2P13, resulted in transformed anthurium plantlets of UH965 and UH1060, based on the following criteria: kanamycin resistance in regenerated plantlets, PCR amplification of NPTII and antibacterial genes (*Att* from UH965 and UH1060, *P13* from UH1060), and western blot detection of attacin protein

in kanamycin-resistant callus derived from regenerated UH965 plantlets. The amplified *Att* gene fragment from DNA of UH965 and UH1060 has the same mobility as the gene fragment amplified from authentic plasmid pCa2Att. Southern blot hybridization also confirmed the amplified DNA to be the *Att* fragment. A *P13* gene fragment was amplified from DNA of putatively transformed UH1060 carrying Ca2P13. The selection marker, *nptII* gene was amplified to an expected size. Southern blot hybridization also confirmed the identity of the amplified gene fragment. PCR analysis of DNA from kanamycin-resistant UH965 from treatment with pCa2T4 did not show evidence of T4 *e* gene integration. Previous work using wild type *A. tumefaciens* for cocultivation with etiolated internodes showed that the monocot anthurium is amenable to *Agrobacterium*-mediated transformation (Kuehnie & Sugii, 1991b). From the present work, it is concluded that transgenic anthurium plants were obtained following treatments with disarmed *Agrobacterium*.

Earlier cocultivation experiments (Experiment 1 to 4) incubated explants in the dark for a long time (more than one year) before regenerating green shoots. In the later experiment, the treated explants were incubated under the low light condition, which allowed faster shoot regeneration without too much callus proliferation. Using this scheme, kanamycin-resistant plantlets of anthurium UH965 and UH1060 were obtained within one year.

The fact that root explants can regenerate shoots (Chapter 3) offers an alternative material for cocultivation with *Agrobacterium*. Shoot formation after cocultivation was observed in the commercial cultivar 'Anuenue'. It will take some time to see whether there are any kanamycin-resistant plantlets. The condition of selection, eg. kanamycin concentration, should

be reassessed to allow faster callus formation and subsequent regeneration. Root explants of *Arabidopsis thaliana* have been successfully used for cocultivation with *Agrobacterium* and subsequent high frequency regeneration of transgenic plants (Huang & Ma, 1992; Márton & Browse, 1991; Valvekens et al., 1988).

GUS activity was never detected in tested anthurium leaf and root tissues. In wheat plants transformed by particle bombardments, the frequency of GUS positive plants was low (Vasil et al., 1992). Although the *gus* gene was detected in genomic DNA blots of four selected transformed plants, no GUS positive staining in the transgenic wheat plants was indicated. This could be due to modifications at the gene or protein level, if the GUS gene was indeed integrated into the plant genome. When GUS was fused with a signal peptide sequence of patatin, the fused protein was transported into the endoplasmic reticulum and modified by glycosylation. The enzymatic activity of the resulting glycosylated protein was inhibited (Iturriaga et al., 1989). Thionins (plant metabolites) have been shown to inactivate GUS activity in both purified enzyme and in protoplasts of transgenic tobacco expressing GUS (Diaz et al., 1992). Suppose *gus* gene is integrated into anthurium genome, it is possible to test whether the GUS enzyme can be expressed by using western blot analysis with anti-ß-glucuronidase antibody. If GUS is not detected in the protein extract, the inactivation of GUS probably is due to modification at the gene level. One can also apply glycosylation inhibitors such as tunicamycin (Cordewener et al., 1991; Elbein, 1981) to plant regeneration medium, to see if the GUS activity in the regenerated kanamycin-resistant plantlets is inhibited due to glycosylation.

The attacin protein expressed in transgenic anthurium plants has the same apparent molecular weight as the standard isolated from insects, which indicates that no processing of the protein was made in anthurium.

Challenging the putatively transgenic anthurium plantlets (from cocultivation of *A.* tumefaciens LBA4404 carrying pCa2Att) with the blight bacteria, *X. campestris* pv. *dieffenbachiae* strain D150, showed a certain degree of resistance to the bacteria in most putatively transformed plants. A second challenge of UH965 transformed with pCa2Att, and UH965 putatively transformed with pCa2P13 or pCa2T4 also showed partial resistance to the bacteria. Some of the small water-soaked spots on kanamycin-resistant Ca2Att anthurium plants from the first challenge inoculation indicated that they were infected by the bacteria. However, these Ca2Att anthurium and other symptomless plants apparently limit bacterial multiplication, since they had fewer bacteria counts in petiole pieces after reisolation than untransformed control plants. In other words, partial resistance was acquired by those putatively transformed UH965 plants. Most of the control anthurium plants were heavily infected by the bacteria. These plants showed either shoot-root rot or shoot wilting, which probably was due to clogging of the vascular system in anthurium plants by the systemic spread of the pathogenic bacteria.

In conclusion, transgenic anthurium plants carrying antibacterial genes were obtained by *A. tumefaciens*-mediated transformation of UH965 and UH1060 internode and lamina explants. Growth chamber challenge studies with some transformed or putatively transformed UH965 plants carrying Ca2Att, Ca2P13 or Ca2T4 implied that they have partial resistance or tolerance to the blight bacteria *Xcd*150.

Further work is necessary to test whether these transgenic anthurium plants are resistant to the blight bacteria in the glasshouse and screenhouse conditions similar to those in which most growers produce their anthurium crop.

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CHAPTER 5

GENETIC TRANSFORMATION OF ANTHURIUM VIA MICROPROJECTILE BOMBARDMENT

5.1 INTRODUCTION

Microprojectile bombardment, or the biolistic process, was developed five years ago (Sanford et al. 1987), as a method to introduce foreign genes directly into cells or tissues in order to obtain transgenic plants. During the ensuing period, other similar devices were also developed, including the electric discharge (Christou, 1990; Christou et al. 1988; McCabe et al. 1988), and compressed air accelerators (Morikawa et al. 1989). Stably transformed plants have been obtained for the following crops using the biolistic process: maize (Gordon-Kamm et al., 1990), wheat (Vasil et al. 1992), rice (Christou et al., 1991), soybean (Christou et al., 1989; Finer & McMullen, 1991; Finer et al., 1992; McCabe et al., 1988), papaya (Fitch et al., 1990), dendrobium orchid (Kuehnle & Sugii, 1992), cranberry (Serres et al., 1992), yellow poplar (Wilde et al., 1992).

In this chapter, various anthurium explants, including etiolated internodes and embryogenic calli, were used as targets for DNA-coated microprojectiie bombardment. Transient expression assays of NPTII and GUS activities and plant regeneration from embryogenic calli are presented.

5.2 MATERIALS AND METHODS

5.2.1 Plasmids

The plasmids used in this study are listed in Table 5-1. pBI121 was obtained from Clontech (Palo Alto, California). pBI426 was obtained from W. L. Crosby (Saskatoon, Canada). It contains a fusion protein of GUS and NPTII (Datla et al., 1991), whose gene is driven by a double CaMV35S promoter followed by a leader sequence of alfalfa mosaic virus (Russell et al., 1992). pSKH501 was obtained from Dr. J. Sanford (Cornell University). It contains the reporter gene *lacZ*, driven by CaMV35S promoter, and has the antibiotic marker NOS/NPTII.

The following antibacterial gene constructs were made by Destéfano-Beltrán (1991) and provided by J. M. Jaynes (Louisiana State University). pCa2C38 contains the insect antibacterial gene C38 (modified cecropin B), driven by the double CaMV35S promoter and inserted into the HindIII site of pBI121. pCa2Att contains the insect antibacterial gene encoding attacin, driven by the double CaMV35S promoter. pWIShiva contains a modified cecropin gene, *Shiva-1*, driven by the wound inducible proteinase inhibitor II promoter of potato (Keil et al., 1989). Both chimeric genes (*Att* and *Shiva-1*) were inserted into the HindIII site of pBI121. The plasmids with pBI121 as backbone all have the reporter CaMV35S/GUS gene and the selectable marker NOS/NPTII gene. The plasmid pMON530-Shiva contains the *Shiva-1* gene, driven by a CaMV35S promoter, and inserted into pMON530 (Rogers et al., 1987). The plasmid also has nopaline synthase gene (*nos*) as a reporter, and NOS/NPTII as selectable marker (Table 5-1).

Table 5-1. Plasmid vectors used for microprojectile bombardments

Plasmid	Promoter /reporter	Promoter /antibiotics	Promoter /gene insert	Reference
pBI121	CaMV35S/GUS	NOS/NPTII	--	Jefferson et al., 1987
pBI426	Ca2-AMV/GUS::NPT fusion		--	Russell et al. 1992
pSKH501	CaMV35S/lacZ	NOS/NPTII	--	J. Sanford[a]
pCa2C38	CaMV35S/GUS	NOS/NPTII	Ca2/Cecropin B	Destéfano-Beltrán, 1991
pCa2Att	CaMV35S/GUS	NOS/NPTII	Ca2/Attacin E	Destéfano-Beltrán, 1991
pWIShiva	CaMV35S/GUS	NOS/NPTII	PiII[b]/Attacin E	Destéfano-Beltrán, 1991
pMON530-Shiva	NOS/Nopaline	NOS/NPTII	CaMV35S/Shiva-1	J. Jaynes[a]

[a]Personal communication
[b]Proteinase inhibitor II promoter of potato

Some of the binary vectors were prepared either by small scale culture of transformed *E. coli* in liquid LB medium (Maniatis et al., 1982) containing 50 μg/ml kanamycin or further purified by cesium chloride density gradient ultracentrifugation (Maniatis et al., 1982).

5.2.2 Plant materials and culture conditions

The following Univ. of Hawaii anthurium selections and cultivars were used in this study: UH965 (now 'Rudolph'), UH1003, UH1060, 'Kaumana', and 'Mauna Kea'. Tissue culture media used in this study were listed in Table 5-2. Etiolated shoots were obtained from node or lamina culture in H1 medium (Kunisaki, 1980; Kuehnle & Sugii, 1991) under the dark. Leaf laminae of UH1003 were excised and cultured in C medium (Table 4-1) for 1 month before particle gun treatment. Petiole explants of UH965 were also 'precultured' on C medium to initiate callus. Etiolated internode explants of UH965 were precultured horizontally on Cmod medium for 2 months to induce callus and shoot formation in the terminal cut surface. At this stage, the shoots were very small on the surface of calli. Embryogenic calli of UH1060 were induced by culturing lamina explants on C medium for 2 to 4 months. Single somatic embryos and small embryogenic calli were removed with sharp scalpel one day before particle bombardment and placed in the center of a 10-cm plastic petri plate containing C medium solidified with 1.5% Difco Bacto-Agar.

In the first bombardment experiment, calli of UH965, UH1003 and UH1060 were maintained on BMS (UH965, 4 plates; UH1003, 2 plates), Cmod (UH1060, 7 plates), D (UH965, 2 plates; UH1003, 3 plates), Dmod

Table 5-2. Composition of media used in tissue culture of anthurium

Components	Medium and composition				
	D	Dmod	BMS	Su	Cmod
Macronutrients	1/2x MS	1/2x MS	MS	MS	1/2x MS
Micronutrients	MS	MMS[a]	MS	MS	MMS[a]
NaFeEDTA	36.7 mg/l	43 mg/l	43 mg/l	43 mg/l	43 mg/l
Vitamins	As MS[b]	As MS[b]	BMS[c]	T[d]	As MS[b]
Sucrose	20 g/l	30 g/l	20 g/l	40 g/l	30 g/l
Glucose	10 g/l	--	--	--	--
Myo-inositol	100 mg/l	--	200 mg/l	--	--
2,4-D	2 mg/l	2 mg/l	2 mg/l	1 mg/l	0.08 mg/l
BA	--	--	--	--	1 mg/l

[a]Same as MS, except H_3BO_3, $MnSO_4$ as 1/2x MS
[b]With 0.4 mg thiamine·HCl/l
[c]BMS vitamins: 1.3 mg/l nicotinic acid, 0.25 mg/l each of thiamine·HCl, pyridoxine·HCl and calcium pantothenate
[d]T, thiamine·HCl only, 0.1 mg/l

(UH1060, 9 plates) media. The media, solidified with 1.5% Difco Bacto-agar for use during particle bombardment only, were dispensed to 10-cm plastic petri plates. Thirty callus pieces (about 0.5 x 0.5 cm each) were centrally placed on the medium in each dish. A total of 28 plates from three cultivars (UH965, UH1003 and UH1060) were bombarded for a total of 840 pieces. Bombarded calli were subcultured every month by division and by cutting into smaller pieces, and were transferred onto freshly prepared media. The calli were cultured at 23-25^{o}C in the dark.

In the second bombardment experiment, three different tissues of four cultivars were used for bombardment with various plasmids. Etiolated internode explants of 'Mauna Kea' cultivar were bombarded with pBI426 (29 internodes, 1 plate), pWIShiva (30 internodes, 1 plate), and pMON530-Shiva (30 internodes, 1 plate). Etiolated internode explants of 'Kaumana' cultivar were bombarded with pBI426 (30 internodes, 1 plate), pWIShiva (31 internodes, 1 plate), and pMON530-Shiva (30 internodes, 1 plate). Etiolated internodes of UH965, precultured on Cmod medium (Table 5-2) for one month, were bombarded with pBI426 (90 internodes, 3 plates), pWIShiva (90 internodes, 3 plates), pMON530-Shiva (118 internodes, 4 plates). Another plate containing 30 precultured UH965 internodes was bombarded with uncoated microprojectiles as a control. Leaf laminae of UH1003, precultured in C medium (Table 4-1) for one month, were bombarded with pBI426 (20 laminae, 1 plate), pWIShiva (19 laminae, 1 plate), and pMON530-Shiva (25 laminae, 1 plate). Two days after microprojectile bombardment, arbitrarily selected explants were used for the GUS transient expression assay. The remaining explants were transfered to Cmod medium containing 50 μg/ml kanamycin except laminae of UH1003

were transferred into liquid C medium containing 50 μg/ml kanamycin. The laminae explants of UH1003 were discarded after two months of culture due to tissue browning and the release of a browning substance into the medium. The bombarded etiolated internodes of cultivars 'Mauna Kea' and 'Kaumana' were either contaminated by fungi or turned brown after 3 months of culture. They were discontinued for further subculture. Etiolated shoots developed from bombarded UH965 internodes, previously cultured in Cmod medium, were transferred onto H1 medium (Table 3-1) containing 50 ug/ml kanamycin in GA-7 boxes. They were cultured in weak light (4 μE $m^{-2}sec^{-1}$, 16 hours light/ 8 hours dark cycle) to allow transition into green shoots.

The third set of bombardments were conducted at HSPA (Aiea, Hawaii) using DuPont PDS-1000 particle gun. Etiolated internodes of UH965, petiole explants of UH1060 previously cultured in C medium (Table 3-1), and embryogenic calli of UH1060 were bombarded with pBI121 (one plate each of UH965 internodes and UH1060 petiole explants; 2 plates of UH1060 calli), pWIShiva (two plates of UH965 internodes; 4 plates of UH1060 petiole explants; and 2 plates of UH1060 calli) and uncoated microprojectiles (one plate each of UH965 intenodes and UH1060 petiole explants; 2 plates of UH1060 calli). Each plate contained 30 pieces of explants centered in a 10-cm plastic petri dish containing Cmod medium solidified 1.5% Difco Bacto-Agar (for particle bombardment only). Twenty-four hours after bombardment, arbitrarily selected explants were assayed for transient GUS expression by histochemically staining according to McCabe et al. (1988). All other explants were transferred to Cmod medium containing 50 μg/ml kanamycin (UH965 internodes) or C medium containing

the same kanamycin (UH1060 petioles and calli) and cultured in the dark at 23-25^{o}C. The bombarded UH965 internodes and UH1060 petioles were discontinued from culturing without waiting for regeneration. The embryogenic calli of UH1060 were moved to weak light (4 $\mu Em^{-2}sec^{-1}$, 16 hours light/ 8 hours dark cycle) after one month of dark culture. The regenerated green shoots were subsequently transferred onto H1 medium (Table 3-1) containing 50 μg/ml kanamycin and cultured at 25^{o}C in higher light condition (32 $\mu Em^{-2}sec^{-1}$, 16 hours light/ 8 hours dark cycle) for root formation.

Tobacco 'Su' cells were also included in the third set of bombardments to check for GUS transient expression. The cell line 'Su' (a gift from Dr. E. D. Earle, Cornell University) was subcultured in liquid Su medium (Table 5-2) at weekly intervals in the dark at 23-25^{o}C. On the day of bombardment, the tobacco suspension cells were removed from liquid medium and spread centrally onto a 5.5-cm sterile S/P filter paper (Baxter Healthcare Corp., McGaw Park, Illinois). The filter paper with the tobacco cells was placed in a 6-cm plastic petri plate containing Su medium solidified with 1.5% Difco Bacto-Agar.

5.2.3 DNA preparation and particle bombardment

The first set of bombardments were performed in J. C. Sanford's laboratory at Cornell University by A. R. Kuehnle and N. Sugii. The second set of bombardments were done in the same lab except the plant materials were sent from Hawaii to J. C. Sanford by mail and returned to Hawaii for analysis. These two experiments used the prototype particle gun. The third

set of bombardments were done at the Hawaii Sugar Planters' Association (HSPA) in Aiea, Hawaii, using gunpowder driven Biolistic PDS-1000 particle gun (DuPont, Wilmington, Delaware).

The microparticle coating procedure is as follows: 50 μl DNA (1 μg/ul) was added to 130 mg tungsten particles (average diameter 1 um) sterilized previously in 70% ethanol and vortexed for 2-3 seconds. 500 μl of ice-cold calcium chloride (2.5 μM) was added and vortexed for 2-3 seconds. 200 μl ice-cold spermidine (0.1 M) was added and vortexed for 10 minutes. The mixture was pulse-centrifuged in a microcentrifuge and the supernatant was removed and discarded. Five μl (or 2.5 μl for the third experiment) of the resulting suspension was placed on the center of each macroprojectile. DNA coated particles were accelerated by gun powder, and hit around the center of the petri plates containing anthurium tissues or tobacco cells. After particle bombardment, the tissues were transferred to freshly prepared media containing 50 μg/ml kanamycin and incubated in the dark at 25^{o}C. Tobacco cells from the third set of bombardments, sitting on the S/P filter paper, were used for transient expression assays directly and not subcultured.

5.2.4 Transient expression assay for GUS activity

Tobacco cells with the filter paper were transferred to empty 6-cm petri plates and X-gluc/buffer (Chapter 4) (McCabe et al., 1988) was added to the plates. They were incubated at 37^{o}C overnight. Any blue spots were counted using a dissecting microscope. Seven days after the first set of bombardments, and 2 days after the second set of bombardments, and 24

hours after the third set of bombardments, arbitrarily selected numbers of explants were stained for GUS expression. Anthurium tissues (laminae, internodes, and embryogenic calli) were placed in wells of 96-well microtiter plates (Falcon 3072, Becton Dickinson Labware, New Brunswick, New Jersey) and x-gluc/buffer added to each well. Plates were incubated at 37^{o}C for 48 hours and blue spots counted using a dissecting microscope.

5.2.5 NPT II activity assay

NPT II assay of materials from the first bombardment experiment followed the protocol of McDonnell et al. (1987), except the γ^{32}P-ATP stock was 26.6 μCi/μl. Briefly, 90 to 210 mg of bombarded and non-bombarded calli of UH1003 and UH1060 were used for extracting proteins. Twenty μl of cleared supernatant of each sample was spotted onto Whatman P81 (cellulose phosphate) paper disc (diameter 2 cm). The paper discs were counted in plastic counting vials containing distilled water with Beckman liquid scintilation counter. After counting, the discs were exposed to X-ray film at -80^{o}C for 5 days and developed. The protein concentration in the extracts was determined by Bradford's method (Bradford, 1976) using Bio-Rad Protein Assay Kit II (Bio-Rad, Cat. No. 500-0002, Richmond, California).

5.2.6 Plating of explants and plant regeneration

Bombarded calli (with DNA or microprojectiles) from the first experiment were transferred to various media (Table 5-1) with 50 μg/ml kanamycin and incubated in the dark at 25^{o}C. For subculture, large pieces of calli were

divided with a scalpel and plated on the same media. However, after several passages, all the calli turned brown. They were discarded without further characterization.

Bombarded internodes and laminae from the second experiment were transferred to fresh C medium (Table 3-1) with 50 μg/ml kanamycin and incubated in the dark at 25^{o}C. However, most of the explants turned brown and died after 2-3 weeks of culture. Others were contaminated by fungi. Only precultured UH965 internodes and UH1060 embryogenic calli (third experiment) survived. The calli were subcultured in 10-cm plastic petri dishes containing Cmod (UH965 precultured internodes) or C medium (UH1060 embryogenic calli), plus 50 μg/ml kanamycin in both cases, and incubated in weak light (4.4 $\mu Em^{-2}sec^{-1}$, 16 hours light/8 hours dark cycle) at 25^{o}C for shoot regeneration. Regenerated shoots from embryogenic calli were transferred to hormone-free H2 medium (Table 4-1) with 50 μg/ml kanamycin for root formation in GA-7 boxes. At this time, they were moved to higher light condition (32-34 $\mu EM^{-2}sec^{-1}$) for further growth and for evaluation of kanamycin resistance. Plants were considered sensitive to kanamycin if newly emerged leaves were either bleached or little or no growth over time observed (very small new leaf). Based on this, the number of sensitive and putative resistant plantlets were scored.

5.3 RESULTS AND DISCUSSION

5.3.1 Expression of GUS activity

In the first set of bombardments, no blue spots were detected in UH965 calli bombarded with either pBI121 or pCa2C38 (Table 5-3). In UH1003 calli bombarded with pBI121, 3 out of 6 pieces had blue spots. No blue spots were detected in pCa2Att- or pCa2C38-bombarded UH1003 calli (Table 5-3). In UH1060 calli, 4 out of 25 pieces of pCa2Att-treated calli had blue spots. Three out of 21 pieces of pCa2C38-treated calli had blue spot. All observed spots were concentrated in tiny discrete centers.

In the control calli bombarded with pSKH501, no blue spots were detected in UH965 and UH1003. Three out of 20 callus pieces of UH1060 had blue spots (Table 5-3). However, these spots are more diffused than those observed in the above-described blue spots. Since pSKH501 does not contain GUS reporter gene, pSKH501-treated UH965 and UH1003 calli and uncoated microprojectile-treated UH965 calli did not show blue spots. The diffused spots found in UH1060 could be due to unknown source.

The second experiment used UH965, UH1003, 'Mauna Kea' and 'Kaumana' tissues for bombardment. In 'Mauna Kea', 44% of the assayed internodes produced blue spots following bombardment with pBI426, and the average number of spots was 2 per internode expressing GUS activity (Table 5-4). Seventeen percent of the assayed internodes produced blue spots following bombardment with pWIShiva, and the average number of spots was 2 per internode (Table 5-4). An example of the blue spots produced after GUS histochemical staining in the bombarded internode is

Table 5-3. Expression of ß-glucuronidase (GUS) activity in anthurium callus pieces 7 days after bombardment with microprojectiles coated with various plasmids

Cultivar	Plasmid	No. treated (# plates)	No. assayed[a]	No. showing blue spots
UH965	pBI121	30 (1)	4	0
	pCa2C38	90 (3)	12	0
UH1003	pBI121	30 (1)	6	3 (50%)
	pCa2Att	30 (1)	4	0
	pCa2C38	60 (2)	9	0
UH1060	pCa2Att	180 (6)	25	4 (16%)
	pCa2C38	150 (5)	21	3 (14%)
Controls				
UH965	pSKH501[b]	60 (2)	8	0
	MP[c]	30 (1)	2	0
UH1003	pSKH501	30 (1)	4	0
UH1060	pSKH501	150 (5)	20	3

[a]GUS histochemical staining according to McCabe et al. (1988) (as in Table 5-4)
[b]The plasmid pSKH501 does not carry the GUS gene
[c]Uncoated microprojectiles only

Table 5-4. Summary of GUS activity detected in anthurium tissues two days following bombardment of microprojectiles coated with different plasmids[a]

Cultivar	Plasmid	No. treated (# plates)	No. assayed	At least one spot		Average No. blue spot per tissue
				Number	%	
Mauna Kea[b]	pBI426	29 (1)	16	7	44	2
	pWIShiva	30 (1)	6	1	17	2
	pMON530-Shiva	30 (1)	ND[f]	-	-	-
Kaumana[b]	pBI426	30 (1)	6	5	83	1
	pWIShiva	31 (1)	6	1	17	3
	pMON530-Shiva	30 (1)	ND	-	-	-
UH965[c]	pBI426	90 (3)	12	2	17	1
	pWIShiva	90 (3)	18	0	0	0
	pMON530-Shiva	118 (4)	ND	-	-	-
	MP[e]	30 (1)	6	0	0	0
UH1003[d]	pBI426	20 (1)	5	0	0	0
	pWIShiva	19 (1)	5	0	0	0
	pMON530-Shiva	25 (1)	5	0	0	0

[a]GUS histochemical staining according to McCabe et al. (1988), examined at least 18 hours after activity sta
stainining at 37°C
[b]Etiolated internodes
[c]Calli with shoots from etiolated internodes precultured in Cmod medium
[d]Leaf laminae precultured in C medium
[e]Uncoated microprojectiles only
[f]Not determined since the plasmid pMON530-Shiva does not contain GUS gene

shown in Fig. 5-1. In 'Kaumana', 83% of the assayed internodes produced blue spots following bombardment with pBI426, and the average number of spots was 1 per internode (Table 5-4). Seventeen percent of the assayed internodes produced blue spots following bombardment with pWIShiva, and the average number of spots was 3 per internode (Table 5-4). In UH965, 17% of the assayed internodes produced blue spots following bombardment with pBI426, and the average number of spots was 1 per internode. No blue spots were detected following bombardment of UH965 with pWIShiva or microparticles only (Table 5-4). In UH1003, no GUS activity was detected in any of the bombarded lamina explants assayed (Table 5-4).

Since the sample size of the assayed explants was too small, it was not possible to compare the differences statistically. From the results of these two particle bombardment experiments, the expression of GUS activity as measured by histochemical staining, varied among genotypes and among different plasmids per genotype. In particular, it appeared that the GUS::NPTII fusion protein had more chance of expression in anthurium, since its transient expression activity could be detected in 3 out of 4 cultivars. The nontranslated leader sequence of alfalfa mosaic virus inserted in PBI426 had been shown to enhance the strength of double CaMV35S promoter (Datla et al., 1991). This fusion protein may be useful for selection and scoring of stably transformed anthurium tissues. Other enhancer-like elements were also reported as useful in plant transgene expression, including 5'-leader sequence of potato virus X (Zelenina et al., 1992) and pea seedborne mosaic potyvirus (Nicolaisen et al., 1992), and intron 1 of the maize *Shrunken-1* (*Sh1*) gene (Maas et al., 1991).

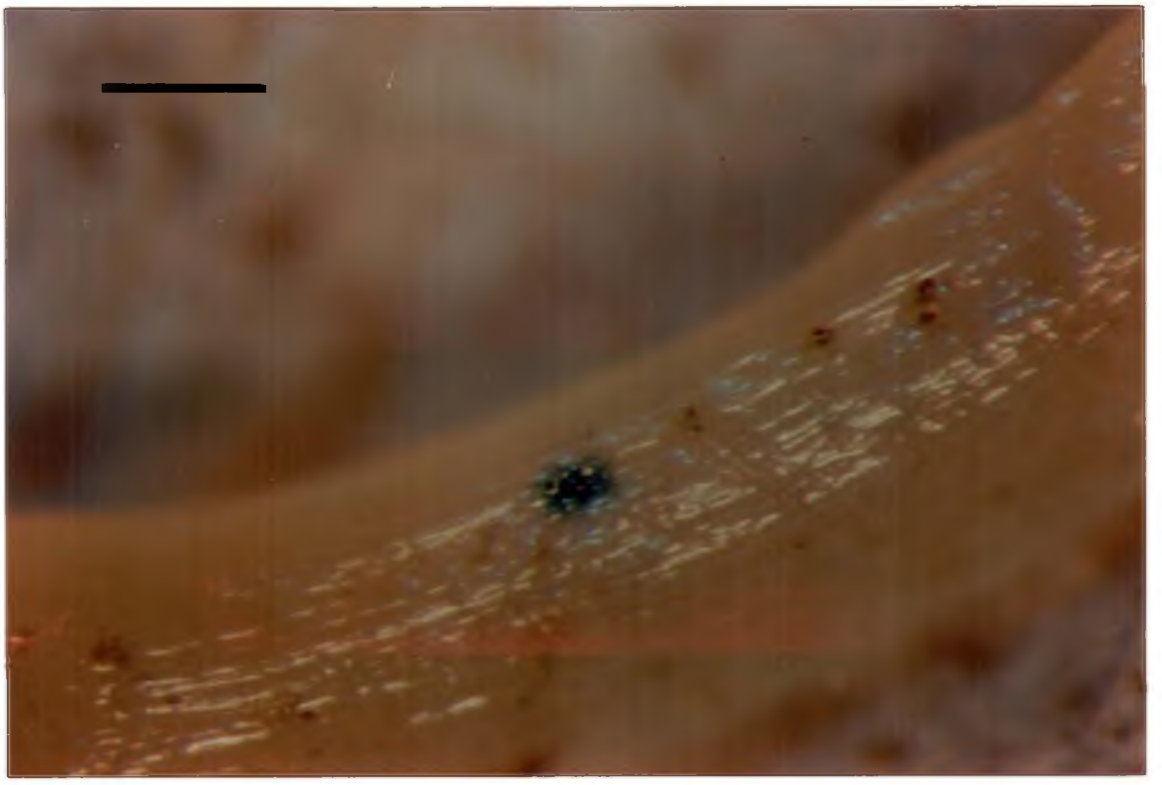

Fig. 5-1. Histochemical analysis of transient expression of ß-glucuronidase activity in the etiolated internode of 'Mauna Kea' bombarded with microprojectiles carrying pBI426. (Bar = 0.4 mm)

In the third experiment, the tobacco 'Su' cell line produced 39 spots in the plate following bombardment with pBI121. No spots were detected in the treatment with pWIShiva (Table 5-5). Since both plasmids have the same GUS reporter, the reason for non-expression in pWIShiva treated cells is unknown. In contrast, transient expression of GUS was detected in anthurium UH965 petiole bombarded with pWIShiva. A total of 5 spots were detected in 24 assayed UH965 petiole explants (Table 5-5). In the treatment of UH965 with uncoated microprojectiles or with pBI121, no blue spots were detected. Among UH1060 internodes, the opposite result was observed. No blue spots were detected in pWIShiva treated explants, while one spot was observed out of 6 assayed internodes (Table 5-5).

In embryogenic calli of UH1060, calli bombarded with pBI121 produced 1 blue spot from 7 assayed pieces. Calli bombarded with pWIShiva produced 3 blue spots from 26 assayed pieces (Table 5-5).

Apparently, GUS expression in these tissues (internodes, petiole, and embryogenic calli) was very low. The site of GUS expression usually is in the epidermal layers, such as the one shown in Fig. 5-1. Since the callus was produced from the pith region of the internodes (chapter 3) and petiole, the potential of regenerating shoots from bombarded internode or petiole explants is expected to be very low. The etiolated internodes or petioles appears to be a good system to test whether a promoter (other than CaMV35S or wound inducible promoter) functions in anthurium tissue. A cloned promoter can be fused to the coding region of the GUS gene and bombarded into anthurium explants to see whether the GUS gene can be expressed in anthurium tissue.

Table 5-5. Transient ß-glucuronidase expression assays 48 hours after microprojectile bombardment of tobacco and anthurium tissues

Plant sample	Plasmid	No. of explant (# plate)	No. assayed[a]	No. explant with blue spots
Control				
Tobacco 'Su' cells	pBI121	NA[c] (1)	1 plate	39
	pWIShiva	NA (1)	1 plate	0
Anthurium				
UH965 internodes	MP[b]	26 (1)	6	0
	pBI121	30 (1)	6	0
	pWIShiva	60 (2)	24	5
UH1060 petioles	MP	30 (1)	6	0
	pBI121	30 (1)	6	1
	pWIShiva	120 (4)	12	0
UH1060 embryogenic callus	MP	60 (2)	8	0
	pBI121	60 (2)	7	1
	pWIShiva	60 (2)	26	3

[a]GUS histochemical staining according to McCabe et al. (1988) (as in Table 5-4)
[b]Uncoated microprojectiles only
[c]Non-applicable

5.3.2 Expression of NPTII activity

NPTII activity was assayed 2 months after bombardment in the first experiment. In UH1003 treated with pCa2Att, NPTII activity was detected by autoradiography (5 day-exposure) and scintillation counts (Table 5-6). Other UH1003 samples did not produce an image on the x-ray film. Their average count of 57 cpm/μg protein was close to the control count of 59 cpm/μg protein (Table 5-6). In UH1060 calli treated with pSKH501, two samples showed radioactivity on the autoradiogram, with an average count of 123 cpm/μg protein. One sample each from pCa2C38- and pCa2Att-treated calli had detectable radioactivity on the autoradiogram, their counts were 109 and 58 cpm/μg protein, respectively (Table 5-6). No UH1060 control count was available for comparison.

5.3.3 Regeneration of multiple shoots from embryogenic calli

From the second bombardment experiment, shoots of UH965 were regenerated in weak light on H1 medium containing 50 μg/ml kanamycin following bombardment. When rooted shoots in this selection condition were moved to higher light, all the newly emerged leaves were bleached in two passages of selection medium.

When pWIShiva DNA bombarded UH1060 embryogenic calli were incubated under low light condition for long time, multiple shoots formed on selective medium (Fig. 5-2). It took about 10 months to regenerate shoots from embryogenic calli. These shoots were transferred to GA-7 boxes containing H2 medium with 75 μg/ml kanamycin, and cultured under higher

Table 5-6. Activity of the marker gene, NPTII, in anthurium callus two months after bombardment with microprojectiles coated with various plasmid DNAs

Cultivar	Plasmid	NPTII activity[a] (cpm/ug protein)	Autoradiogram[b]
UH1003	NA[c]	59	-/-
	pCa2C38	57	-/-
	pCa2Att	67	+/-
UH1060	pSKH501	123	+ +/+
	pCa2C38	109	-/+
	pCa2Att	58	+/-

[a]Average of two independent samples
[b]Image of individual samples. +, mild activity; + +, stronger activity; -, no activity
[c]Unbombarded control

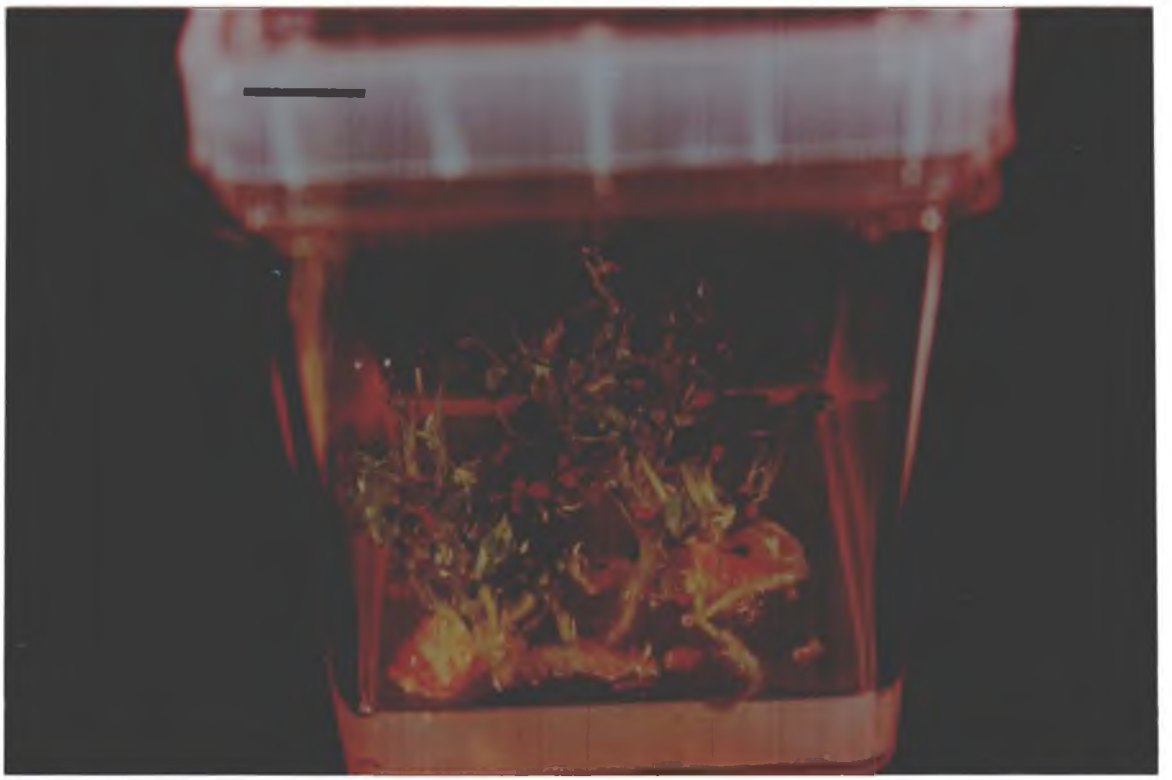

Fig. 5-2. Regeneration of shoots from embryogenic calli of UH1060 anthurium on selective medium in weak light after microprojectile bombardment. (Bar = 10 mm)

light condition at 25°C. From 2 plates, a total number of 1870 plantlets were regenerated. Upon subculture onto kanamycin-containing medium, they were all found to be kanamycin-sensitive, except one which appeared to be kanamycin-resistant (Table 5-7), since the newly emerged leaf of this putative kanamycin-resistant plantlet remained green. These plantlets grew very slowly on kanamycin-containing medium, which made the evaluation of kanamycin resistance difficult. However, upon transfer to fresh medium with 75 μg/ml kanamycin every 2-3 months, kanamycin-sensitive plants could be identified by their bleached newly-emerged leaves. These plants were then discarded. The only putative kanamycin-resistant plantlet is not yet big enough for molecular characterization.

From the above number of regenerated plantlets, it appears that embryogenic callus is a better target than other explant sources, including etiolated internodes, petioles and laminae, for particle bombardment, although the efficiency is too low. When microprojectile bombardment is compared to *Agrobacterium*-mediated transformation, the latter is the preferred method of transferring genes into anthurium, since only one putative kanamycin-resistant plantlet was recovered from the former method, while at least 680 kanamycin-resistant UH1060 plantlets were regenerated (Table 4-10) using the latter method. In addition, *Agrobacterium* strains are more accessible and much easier to maintain than microprojectile accelerator.

Embryogenic callus has been successfully used in maize (Fromm et al., 1990; Gordon-Kamm et al., 1990), wheat (Vasil et al., 1992), papaya (Fitch et al., 1990), soybean (Finer & McMullen, 1991), and yellow-poplar (Wilde et al., 1992) for production of transgenic plants following particle

Table 5-7. Recovery of putative kanamycin resistant anthurium (UH1060) regenerated from embryogenic calli following particle bombardment with plasmid pWIShiva

Treatment	No. of calli (# plate)	No. Km-s[a] plantlets	No. putative Km-r[b] plantlets
MP[c]	60 (2)	NR[d]	NR
pWIShiva	34[e] (2)	1869	1

[a]Kanamycin sensitive
[b]Kanamycin resistant
[c]Uncoated microprojectiles only
[d]No regeneration occurred
[e]Original number was 60 calli pieces, 26 were used for GUS transient expression assay (Table 5-5)

bombardment. Finer and McMullen (1991) obtained an average of 709 GUS-positive foci (spots) of embryogenic soybean cells 3 days after bombardment, and the transient-to-stable conversion frequency was about 0.4%. In anthurium callus, 60 pieces of embryogenic callus were used for bombardment with the plasmid pWIShiva (Table 5-7, see footnote). Three out of 26 pieces assayed for transient GUS expression following bombardment showed blue spots (Table 5-5). This means the frequency of transient expression is 12%. If the only one putative kanamycin-resistant plantlet (Table 5-7) is considered a stable transformant, then at least one of the original 34 callus pieces (Table 5-7) will produce a transformed plantlet. Therefore the frequency of stable transformation will be about 3%. From works with several crops discussed above and from this study, it appears that somatic embryos or embryogenic calli are good targets for particle bombardment-mediated transformation.

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APPENDIX A

AGROBACTERIUM-MEDIATED TRANSFORMATION OF *ANTHURIUM* ETIOLATED INTERNODES WITH ANTIBACTERIAL GENES CONTAINING SIGNAL SEQUENCES

A.1 INTRODUCTION

Kanamycin-resistant anthuriums have been obtained through *Agrobacterium*-mediated transformation, (Chapter 4). The expression of the antibacterial protein attacin in the de novo callus from regenerated kanamycin-resistant anthurium was verified by Western blot analysis (Chapter 4). Preliminary challenges of these kanamycin-resistant anthurium plants with the blight bacterium *Xanthomonas campestris* pv. *dieffenbachiae* 150 resulted in some degree of resistance to the bacteria. Since phytopathogenic bacteria are present in intercellular spaces (Billing, 1982), it would be valuable if the antibacterial proteins can be produced and secreted into intercellular space to overcome the intruding bacteria. By using a heterologous protein fusion with the signal peptide sequence of either a pathogenesis-related protein or an insect cecropin, it was shown that the proteins were secreted into the intercellular space of transgenic tobacco by signal peptide-mediated translocation (Denecke et al., 1990). Based on this observation, a series of antibacterial genes were fused to signal peptide sequences (J. M. Jaynes, personal communication) and made available to us. This brief report describes the cocultivation of anthurium internodes with *Agrobacterium*

tumefaciens carrying antibacterial genes *Shiva-1* and *SB37*, fused with either cecropin B signal peptide sequence or signal sequence of pathogenesis-related protein 1b.

A.2 MATERIALS AND METHODS

A.2.1 Plant materials and culture conditions

The University of Hawaii advanced selection UH1060 was used in this experiment. Etiolated internodes were obtained from etiolated shoots by culturing in vitro-grown leaves in the dark on H1 medium containing 0.2 mg/l BA (Kunisaki, 1980; Table 3-1).

A.2.2 Bacteria, plasmids and media

Agrobacterium tumefaciens LBA4404 carrying plasmids of antibacterial constructs (Table A-1) were kindly provided by J. M. Jaynes of Louisiana State University, Baton Rouge. Antibacterial gene *S1* encodes the synthetic modified cecropin B peptides (Shiva-1). Antibacterial gene *B37* encodes another modified cecropin B (SB37) (Jaynes et al. 1988; Destéfano-Beltrán, 1991). Two different signal sequences were ligated to the *S1* and *B37* genes. Plasmids pBCCS1 and pBCCB37 contain a modified signal sequence of cecropin B from *Hyalophora cecropia* (Van Hofsten et al., 1985). This sequence is similar to a modified form of the sCEC sequence reported by Denecke et al. (1990). Plasmids pBPRS1 and pBPRB37 contain signal sequence from the pathogenesis-related protein 1b (sPR1) of tobacco

Table A-1. Properties of the plasmids carrying signal peptide-antibacterial peptide fusions

Plasmid[a]	Promoter /reporter	Promoter /antibiotic gene	Promoter/signal /insert
pBCCB37	CaMV35S/GUS	NOS/NPTII	Ca2MV35S/sCE[a]/SB37
pBCCS1	CaMV35S/GUS	NOS/NPTII	Ca2MV35S/sCE/Shiva-1
pBPRB37	CaMV35S/GUS	NOS/NPTII	Ca2MV35S/sPR[b]/SB37
pBPRS1	CaMV35S/GUS	NOS/NPTII	Ca2MV35S/sPR/Shiva-1

[a]From J. M. Jaynes
[b]sCE, signal sequence of cecropin B
[c]sPR, signal sequence of tobacco pathogenesis-related protein 1b

(Denecke et al., 1990). All the hybrid genes were driven by a double CaMV35S promoter (Kay et al., 1987) and inserted into the *Hind* III site of pBI121 (Jefferson et al., 1987). LB medium with appropriate antibiotics (Miller, 1972) was used either as liquld or solidified with agar for *Agrobacterium* culture.

A.2.3 Cocultivation, selection and regeneration

The conditions for cocultivation are the same as described in Chapter 3. After cocultivation for 2 days on Cmod medium (Kuehnle and Sugii, 1991), the internode explants were transferred to Cmod medium with kanamycin (50 μg/ml) and either carbenicillin (250 or 500 μg/ml) or cefotaxime (250 μg/ml) (selection medium). The explants were sub-cultured on fresh selection medium twice in a two month-period under conditions of darkness at 23-25^{o}C. After two months, the number of explants forming callus was scored. The explants were then transferred to H1 medium containing 50 ug/ml kanamycin and 250 ug/ml carbicillin for plant regeneration under weak light (4 $\mu Em^{-2}sec^{-1}$, 16 hours light/8 hours dark cycle) at 25^{o}C. After 40 days, the samples were transferred to C medium (Table 3-1) containing 250 μg/ml carbenicillin and 50 μg/ml kanamycin and cultured for 40 days. They were then transferred to Magenta GA-7 boxes containing H1 medium with antibiotics in higher light (32 $\mu Em^{-2}sec^{-1}$, 16 hours light/8 hours dark cycle) at 25^{o}C. The number of explants regenerating shoots was scored after a total time of regeneration of 10 months.

Table A-2. Response of UH1060 etiolated internodes six months after cocultivation with *Agrobacterium tumefaciens* LBA4404 carrying various plasmids

Treatment	No. explants cocultured	No. forming callus[a]	No. calli forming shoots (%)
Control[b]	21	3	0 (0)
pBCCS1	42	17	9 (21)
pBPRS1	44	18	5 (11)
pBPRB37	42	18	4 (10)

[a]Scored two months after cocultivation
[b]Treated with LB medium only

A.3 RESULTS AND DISCUSSION

Four different vectors derived from two signal peptide sequences and two antibacterial genes were used in this experiment. One of the cocultivations containing pBCCB37 was contaminated during selection of cocultured internodes. Following cocultivation of internode explants with *Agrobacterium* containing pBCCS1, 21% of the explants produced multiple shoots after 6 months. For treatments with pBPRB37 and pBPRS1, the frequency of regeneration was 10% and 11%, respectively (Table A-2). The regenerated shoots were transferred to hormone-free medium (H2) with 75 μg/ml kanamycin for root formation and cultured in higher light conditions. This experiment showed that under weak light conditions shoot formation could be obtained in a short time (six months). The number of kanamycin-resistant plantlets will be scored after two transfers to H2 medium with kanamycin. Antibody against SB37 is also available for future verification of the SB37 protein expression by Western blot analysis of kanamycin-resistant UH1060 SB37 plants.

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APPENDIX B

AGROBACTERIUM-MEDIATED TRANSFORMATION OF *NICOTIANA TABACUM* CV. TURKISH WITH PLASMIDS CARRYING ANTIBACTERIAL GENES

B.1 INTRODUCTION

Tobacco has been extensively used as target for *Agrobacterium*-mediated transformation. Transgenic tobacco plants can be used to study temporal and spatial expression of reporter or selectable marker genes driven by different promoters (Benfey & Chua, 1989). It is also useful to study expression of introduced novel genes from other than plant origins.

Antibacterial genes from insects, bacteriophages, and other sources have been introduced into plant expression vectors (Destéfano-Beltrán, 1991). To combat the phytopathogenic bacteria in the intercellular spaces, it is desirable to express and secrete introduced antibacterial genes into intercellular spaces. Signal peptide sequences of an insect cecropia and tobacco pathogenesis-related protein have been fused to genes for use in plants (Denecke et al., 1990).

This brief report describes the introduction of antibacterial genes via *Agrobacterium*-mediated transformation of tobacco.

B.2 MATERIALS AND METHODS

B.2.1 Plant materials and culture conditions

Tobacco (*Nicotiana tabacum*) cultivar 'Turkish' was used through the experiments. The first three experiments used leaf explants from seedlings germinated in vitro for cocultivation. The fourth experiment used leaf explants from a pot-grown tobacco plant, which was derived as a fast regeneration clone from seedling leaf culture. Seeds were sterilized in 10% Clorox for 10 minutes with occasional shaking. They were washed twice in sterile water and transferred to 10-cm Petri dish (100 seeds/plate) containing 1/2MSO medium (Table B-1) solidified with 0.7% Difco Bacto-agar. Seeds were germinated under light (32 $\mu Em^{-2}sec^{-1}$, 16 hr light/8 hr dark cycle) at 25^{o}C. Germinated seedlings were transferred to Magenta GA-7 boxes (Sigma Chem. Co.) with the same freshly prepared medium. The leaves from these seedlings served as material for cocultivation with *Agrobacterium*.

On one occasion while culturing seedling leaves from in vitro germinated 'Turkish' seeds, one explant regenerated multiple shoots within one week. This occurred on 1/2MS104, a regeneration medium containing 1 mg/l naphthalene acetic acid (NAA) and 0.5 mg/l benzyladenine (BA) (Table B-1) modified from Horsch et al. (1988). The regenerated shoots were rooted in 1/2MSO medium. One plantlet was transferred to a 6-inch plastic pot containing potting mix. The plant was allowed to flower, hand-pollinated with self pollens, and mature seeds harvested (designated 'fast regenerant Turkish'). One leaf from the pot-grown plant served as source for

Table B-1. Composition of media used in tissue culture and cocultivation of tobacco

	Medium and composition	
Components	1/2MSO	1/2MS104
Macronutrients	1/2MS	1/2MS
Micronutrients	MS	MS
NaFeEDTA	25.7 mg/l	25.7 mg/l
Vitamins	As MS[a]	As MS[a]
Sucrose	30 g/l	30 g/l
Myo-inositol	100 mg/l	100 mg/l
NAA	--	0.1 mg/l
BA	--	1 mg/l

[a]With 0.4 mg thiamine·HCl/l

cocultivation with *Agrobacterium* carrying antibacterial gene constructs with signal peptide sequences. The leaf was sterilized in 10% Clorox containing two drops of Tween-20 with shaking. It was washed twice with sterile water and used for cocultivation.

B.2.2 Bacteria and plasmids

Agrobacterium tumefaciens LBA4404 was used in all cocultivation experiments. The bacteria were cultured in solid or liquid LB medium, pH 7.0. Two groups of plasmid constructs in *Agrobacterium* were used for tobacco transformation. Table 4-1 lists the first group of constructs carrying antibacterial genes, which were driven by a double CaMV35S promoter. Table A-1 lists the second group of constructs carrying antibacterial gene *SB37* with the modified leader sequence either from cecropin B (Denecke et al., 1990; Van Hofsten et al., 1985; J. M. Jaynes, personal communication) or from tobacco pathogenesis-related protein PR-1a (Denecke et al., 1990; J. M. Jaynes, personal communication) driven by a double CaMV35S promoter. These plasmids were all generously supplied by J. M. Jaynes (Louisiana State University, Baton Rouge).

B.2.3 Cocultivation, selection and regeneration

In the first three cocultivation experiments, sterile tobacco leaf explants were cut with a scalpel into about 0.5 x 1 cm pieces. Five ml of overnight cultures of *Agrobacterium tumefaciens* LBA4404 carrying various plasmids were diluted 10-fold with LB medium, and acetosyringone was added to a

final concentration of 20 μM. The leaf explants were immersed in the bacterial suspension and then transferred to 1/2MSO medium. The cocultures were incubated for 2 days in the dark at 23-25^{o}C. Cocultured leaf explants were gently touched on the agar of the same plate to remove most of the bacteria. They were transferred to 1/2MS104 medium containing 500 μg/ml carbenicillin and 300 μg/ml kanamycin. The cocultured leaf explants were incubated under lighted conditions (32 $\mu Em^{-2}sec^{-1}$, 16 hours light/8 hours dark cycle) at 25^{o}C. After two to four weeks, shoots about 0.5 cm long or smaller were excised and transferred to 1/2MSO medium containing 100 μg/ml kanamycin and 250-500 μg/ml carbenicillin for root formation. The shoots were topped and rooted in the same freshly prepared medium in 2 month intervals for a total of four months. The topped shoots were then rooted in 1/2MSO medium with 100 μg/ml kanamycin. The leaves of these kanamycin-resistant plants were used for GUS assays (Section B.2.4).

In the fourth cocultivation experiment, a single leaf from pot-grown fast regenerant 'Turkish' was sterilized and used for cocultivation. The procedures of cocultivation with *Agrobacterium* was the same as previous cocultivations except the regeneration medium 1/2MS104 was used, instead of 1/2MSO.

B.2.4 GUS histochemical staining

Leaf segments from kanamycin-resistant plants were excised and stained for GUS activity. Two kinds of staining buffers were used interchangeably, depending on their availability. The first buffer consisted of 10mM Na_2-

EDTA, 100 mM $NaH_2PO_4.H_2O$, 0.5 mM $K_4Fe(CN)_6.3H_2O$ and 0.1% Triton X-100 (McCabe et al., 1988), pH 7.0. The second buffer was provided by G. L. Nan (A. R. Kuehnle's lab). This buffer was developed by J. Yoder of Univ. of California at Davis. It consists of 100 mM sodium phosphate, pH 7.0, 1% Triton X-100, 1% dimethyl sulfoxide (DMSO), and 10 mM Na_2-EDTA.

5-Bromo-4-chloro-3-indolyl-ß-D-glucuronic acid (X-gluc)(Research Organics, Cleveland, Ohio) was dissolved in DMSO (50 mg per ml) and stored at -20°C until use. For detection of GUS activity in tobacco tissues, 10 µl of X-gluc/DMSO was suspended in 1 ml GUS buffer and used for histochemical staining.

The leaf segments of wild type controls and kanamycin-resistant tobaccos and the substrate-buffer were incubated at 37°C for 4 hours to overnight. To visualize the blue color, 1 ml of 95% ethanol was added to each sample and incubated at 25°C with occasional shaking until the green chlorophyll was bleached out from the leaf segments.

B.2.5 DNA analysis

Genomic DNA was isolated from leaves or seedlings of transgenic tobaccos by a protocol modified from Dellaporta et al. (1983). One to 3 grams of leaves or seedlings were ground in powdered dry ice to a fine powder in a cold mortar with pestle. Fifteen ml of extraction buffer (100 mM Tris-HCl, pH 8.0, 50 mM Na2-EDTA, 500 mM NaCl and 10 mM 2-mercaptoethanol) were added to the powder. The extract was transferred to a 30 ml polycarbonate tube. One ml of 20% Sodium dedecyl sulfate (SDS) was

mixed thoroughly with the extract and incubated at 65^{o}C water bath for 10 minutes. Five ml of 5 M potassium acetate was then mixed and the mixture incubated on ice for 20 minutes. The mixture was centrifuged at 25,000 x g in a Sorval SS-34 rotor at 4^{o}C for 20 minutes. The suernatant was poured through a Miracloth filter (Calbiochem) into a clean tube containing 10 ml of ice cold isopropanol. After mixing gently, the mixture was incubated at -20^{o}C for 30 minutes. Nucleic acids were pelleted by centrifuging at 20,000 x g at 4^{o}C for 30 minutes. The pellet was washed with 1 ml of 70% cold ethanol and air-dried for 5 minutes. The pellet was resuspended in 400 μl TE buffer (10 mM Tris.HCl, 1 mM EDTA, pH 7.5). Five μl of RNase (5 mg/ml) (Sigma Chem. Co.) was added to the suspension. Upon complete resuspension, the solution was extracted with equal volume of phenol-chloroform-isoamyl alcohol (Sigma P-3803). The aqueous phase was extracted with equal volume of chloroform-isoamyl alcohol (24:1). The volume of aqueous phase was adjusted to 400 μl with TE. 1/10 of 3M sodium acetate and 2.5 volumes of cold absolute ethanol were mixed with the solution. The mixture was incubated at -20^{o}C for at least 30 minutes. It was centrifuged at top speed in Eppendorf microcentrifuge at 4^{o}C. The DNA pellet was washed with 70% ethanol twice and air-dried for 2 minutes. DNA pellet was resuspended in 300 μl TE. DNA concentration was calculated by measuring at 260 nm with a spectrophotometer (Spectronic 601, Milton Roy).

For PCR analysis, 0.5 μg of genomic DNA from untransformed or transgenic tobacco were used. The primers for *Att*, *nptII* and *gus* genes and conditions for PCR have been described in Chapter 4. After DNA amplification, 10 μl of each sample were separated in 0.8% agarose gel

(Fisher) in 0.5X TBE (1X buffer: 0.089 M Tris-borate, 0.089 M boric acid and 0.002 M EDTA, pH 8.0) at 47-59 volts. The gel was stained with ethidium bromide and visualized on a UV transilluminator.

Southern blot hybridization of PCR-amplified products was essentially the same as described in Chapter 4. *Att* was amplified from pCa2Att and labeled with digoxigenin-11-dUTP (Boehringer Mannheim) by random priming with Klenow enzyme (Feinberg & Vogelstein, 1983) according to manufacturer's directions.

B.2.6 Genetic analysis of kanamycin resistance in progenies

The regenerated kanamycin-resistant tobaccos were designated as R0 plants. The selfed progeny from R0 were designated R1. R2 seeds were derived from selfed R1. The progeny between two different R0 plants were designated F1. Kanamycin-resistant R1 plants were also backcrossed to untransformed 'Turkish' tobacco. All these progenies were used for studying the inheritance of kanamycin resistance. Seeds were sterilized with 10% Chlorox in a small vial. They were rinsed with sterile water once. One hundred seeds were placed on the surface of 1/2MSO medium containing 200 mg/l kanamycin in a 10-cm plastic Petri plate. The plates were incubated under light condition at 25^{o}C for 3 weeks.

Kanamycin-resistant seedlings have long roots and root hairs, and produce 2 to 4 true leaves. In comparison, kanamycin-sensitive seedlings only have cotyledons and very short roots, or even no roots. Some kanamycin-sensitive seedlings are bleached in the cotyledons. The number

of germinated seedlings and kanamycin-resistant and -sensitive seedlings was scored. The chi square (X^2)value was hand-calculated.

B.3 RESULTS

B.3.1 Kanamycin resistance and GUS expression in regenerated tobacco plants following cocultivation

Regeneration of shoots from cocultured leaf explants took about 4-5 weeks for the first 3 experiments. In the fourth experiment, a fast regenerating line was used for cocultivation. The regeneration of shoots took 2-3 weeks in experiment 4. In 24 days, the shoots were transferred to rooting medium with 250 μg/ml carbenicillin and 100 μg/ml kanamycin. Kanamycin-resistant shoots formed roots readily in this growth regulator-free medium containing kanamycin.

In the first three cocultivation experiments, the time period required for recovery of kanamycin-resistant plantlets and first flower was 5 to 6 months. In the fourth cocultivation experiment, the time period required for recovery of kanamycin-resistant plantlets and first flower was 3 months.

In the first cocultivation experiment, from 45% to 58% of the explants produced kanamycin-resistant shoots (Table B-2). While control explants did not produce any shoots due to bleaching under selection conditions. In the second and third experiments, 61% to 100% of the explants produced kanamycin-resistant shoots (Table B-2). In the fourth experiment, the

Table B-2. Summary of kanamycin resistance and GUS activity in regenerated tobacco 'Turkish' cocultivated with *Agrobacterium* carrying various plasmids

Treatment	No. leaf explant	No. producing Km-r[a] shoots (%)	No. of Km-r with GUS+
Experiment 1			
Control	20	0 (0)	0
pCa2Att	20	9 (45)	8
pCa2P13	17	8 (47)	5
pCa2T4	19	11 (58)	7
pWIAtt	22	11 (50)	10
Experiment 2			
Control	21	0 (0)	0
pCa2Att	23	22 (96)	4
pCa2P13	22	20 (91)	3
pCa2T4	22	21 (95)	1
pWIAtt	23	19 (83)	3
pWIShiva	21	21 (100)	ND[b]
Expreriment 3			
pWIShiva	51	31 (61)	6
Experiment 4			
pBCCB37	20	16 (80)	--[c]
pBPRB37	20	15 (75)	--

[a]Km-r, Kanamycin resistant
[b]Not determined due to contamination
[c]Not determined

frequency of explants producing kanamycin-resistant shoots was 75% and 80% respectively (Table B-2).

GUS activity was detected by histochemical staining in leaf segments of selected kanamycin-resistant shoots. The number of GUS positive shoots differed among experiments (Table B-2). The pattern of GUS expression also differed in different regenerated plantlets. Some leaves had blue color (an indication of GUS activity) restricted to the middle and minor veins, while others showed blue throughout the leaf blade. Some leaves had intense blue, while other leaves were light blue color.

B.3.2 DNA analysis

DNA from leaf samples of selected kanamycin-resistant plantlets and untransformed control plant were subjected to PCR amplification for *Att*, *npt*II, and *gus* genes. The amplified DNA segment had the expected size of 546 bp (*Att*), 1054 bp (*nptII*) and 1724 bp (*gus*), respectively. Southern blot analysis for the amplified *Att* gene fragment showed hybridization to digoxgenin-labelled *Att* probe (amplified from plasmid pCa2Att).

B.3.3 Genetic analysis of kanamycin resistance

In the R1 progenies investigated, 5 plants showed the expected 3:1 segregation ratio for kanamycin resistance. One plant had a segregation ratio of 4:1. Two plants had 10:1 ratio. One plant had 19:1 ratio (Table B-3).

Table B-3. Genetic analysis of R1 generations derived from kanamycin-resistant and GUS positive R0 transgenic tobaccos germinated on selection medium

Plant	No. of seeds tested	No. germinated	Segregation[a] Km-r	Km-s	Ratio	X^2	P
Turkish[b]	305	286	0	286	--	--	--
Ca2Att-1	300	285	264	21	10:1	1.02	0.25<P<0.5
Ca2T4-1	300	296	209	87	3:1	3.04	0.05<P<0.1
Ca2T4-2	400	377	358	19	19:1	0	P>0.95
Ca2T4-5	600	571	415	156	3:1	1.64	0.1<P<0.25
Ca2T4-6	300	296	266	30	10:1	0.39	0.5<P<0.75
Ca2P13-8-3	100	97	73	24	3:1	0	P>0.95
Ca2P13-2-2	202	197	142	55	3:1	0.89	0.25<P<0.5
WlAtt-2-2	398	341	279	62	4:1	0.70	0.25<P<0.5
WlAtt(II)-4-1	300	284	207	77	3:1	0.68	0.25<P<0.5

[a]Km-r, Kanamycin resistant; Km-s, kanamycin sensitive
[b]Untransformed control

Table B-4. Genetic analysis of R2 generations derived from kanamycin-resistant and GUS positive R1 plants

Plant	No. of seeds tested	No. germinated	Segregation[a] Km-r	Km-s
Ca2T4-5	300	1[b]	1	ND
WIAtt-2-2-1	300	287	274	13
WIAtt-2-2-2	300	299	281	18
WIAtt-2-2-3	200	191	175	16

[a]Km-r, kanamycin-resistant; Km-s, kanamycin-sensitive; ND, not determined
[b]Stock plant burned due to growth chamber failer

Table B-5. Genetic analysis of backcross generations between transgenic kanamycin-resistant R1 and untransformed plants

Cross[a]	No. of seeds tested	No. germinated	Segregation[b] Km-r	Km-s	Ratio	X^{2c}
Ca2T4-1 x WT	200	189	105	84	1:1	2.34
WT x WIAtt-2-2	100	97	42	55	1:1	1.74
WT x WIAtt(II)-4-1	200	185	87	98	1:1	0.66
Ca2Att(II)-5 x WT	200	190	88	102	1:1	1.04
WT x Ca2Att(II)-5	200	187	98	91	1:1	0.29

[a]Female x male; WT, untransformed wild type Turkish
[b]Km-r, kanamycin-resistant; Km-s, kanamycin-sensitive
[c]Based on expected heterozygosity for Km-r

Table B-6. Genetic analysis of kanamycin resistance in F1 generations derived from crosses between two different R0 transgenic plants

Cross	No. of seed tested	No. germinated	Segregation Km-r	Km-s
Ca2T4-6 x WIAtt-2-2	200	193	143	50
Ca2T4-1 x WIAtt-2-2	300	283	136	147

R2 progenies were produced from kanamycin-resistant and GUS positive R1 plants. The segregation ratio for kanamycin resistance was shown in Table B-4. The result of Ca2T4-5 plant (Table B-4) could not be determined due to growth chamber failure and burning of most of the seed pods. In 3 different plants derived from 3 WIAtt-2-2 R1 plants, the segregation ratios ranged from 10:1 to 21:1 (Table B-4).

Selected kanamycin-resistant R1 plants were backcrossed to an untransformed parent. The result was shown in Table B-5. All of the tested progenies segregated as 1:1 ratio. This means primary transformants (R0), Ca2T4-1, WIAtt-2-2, WIAtt(II)-4-1, and Ca2Att(II)-5 all contained single insert.

The segregation ratios in progenies of two R0 plants were 3:1 and 1:1, respectively (Table B-6).

B.4 DISCUSSION

The time period for regeneration and flowering of kanamycin-resistant plants following cocultivation of leaf explants of fast regenerant 'Turkish' was shortened to 3 months from 4 to 5 months. The use of regeneration medium (1/2MS104) during cocultivation probably also promotes shoot regeneration from leaf explants. The transformation frequency (kanamycin-resistant shoots) of this experiment is between 75% to 80%. In the cocultivation of *Arabidopsis* cotyledon explants with *Agrobacterium*, the transformation frequency (kanamycin-resistant calli) was increased up to 92% if the explants were precultured for 5 days in culture media with growth regulator.

Without preculture, the transformation frequency was 10% (Sangwan et al., 1992).

PCR analysis indicated the integration of *Att*, *T4* and *P13* genes into the tobacco genome. The amplified fragment sizes were as expected, based on agarose gel electrophoresis. The identity of the amplified *Att* gene was also verified by Southern blot hybridization.

Genetic analysis revealed that *Agrobacterium*-mediated transformation of tobacco resulted in either single insertion (3:1 ratio in R1 and 1:1 ratio in backcrosses, Table B-3, B-5) or multiple insertion (Table B-3). In the WIAtt-2-2 R1 plant, the segregation ratio was 4:1 (Table B-3), which implied multiple insertion of foreign DNA. However, when backcross progeny of WIAtt-2-2 plant was analysed, the result was 1:1 ratio. This discrepency probably might be due to the expression of antibacterial gene *Att*, which somehow affected the seedling growth in kanamycin medium.

This study confirmed the inheritance patterns of kanamycin resistance, which had been previously reported. Heberle-Bors et al. (1988) reported that most T-DNA insertion was into a single site. Others contained multiple independent copies of T-DNA. Budar et al. (1986) have also shown that most of the *Agrobacterium*-mediated transgenic tobacco clones contained a single kanamycin-resistance locus. Other studies using direct gene transfer (PEG treatment or particle bombardment) also indicated single and multiple insertions of introduced kanamycin-resistance gene (Gharti-Chhetri et al., 1990; Potrykus et al., 1985; Tomes et al., 1990).

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APPENDIX C

COCULTIVATION OF ANTHURIUM EXPLANTS WITH *AGROBACTERIUM*

MEDIA AND SOLUTIONS:

Cmod (cocultivation of internodes and callus initiation)(per liter):

MS macro	50 ml
MS micro (Modified[a])	5 ml
MS F(8600 mg/l)	5 ml
MS G	5 ml
Sucrose	30 g
BA (0.2 mg/l)	5 ml
2,4-D (0.5 mg/l)	0.16 ml
Difco Bacto-Agar	7 g

pH 5.6-5.8

[a]Same as MS, except H_3BO_3, $MnSO_4$ as 1/2MS

H1 (cocultivation of laminae and regeneration)(per liter):

MS macro	50 ml
MS micro	10 ml
MS F(7340 mg/l)	5 ml
MS G	2.5 ml
Sucrose	20 g
Myo-inositol (10 mg/ml)	10 ml
BA (0.2 mg/l)	2 ml

Difco Bacto-Agar	7 g

pH 5.6-5.8

H2 (plantlet maintenance)(per liter):

MS macro	50 ml
MS micro	10 ml
MS F(7340 mg/l)	5 ml
MS G	2.5 ml
Sucrose	20 g
Myo-inositol (10 mg/ml)	10 ml
Coconut water	150 ml
Difco Bacto-Agar	7 g

pH 5.6-5.8

C (callus formation and somatic embryogenesis)(per liter):

MS macro	50 ml
MS micro	10 ml
MS F(7340 mg/l)	3.5 ml
MS G	5 ml
Sucrose	20 g
Myo-inositol (10 mg/ml)	10 ml
Kinetin (0.2 mg/l)	2.5 ml
2,4-D (0.5 mg/l)	3 ml
Difco Bacto-Agar	7 g

pH 5.6-5.8

LB medium (per liter):

Bacto-tryptone	10 g
NaCl	5 g
Yeast extract	10 g

Adjust pH to 7.0 with 1 N NaOH

Acetosyringone (AS) stock (0.5 M):

Dissolve 0.25 g of AS in 2.55 ml dimethyl sulfoxide (DMSO)

Filter (0.22 μm) sterilize the solution

Store IN THE FREEZER (-20^{o}C)

PROTOCOL:

1. Pick a single colony of *Agrobacterium tumefaciens* LBA4404 carrying pCa2Att and culture in 5 ml LB containing 50 μg/ml kanamycin and 25 μg/ml streptomycin, 250 rpm, 28^{o}C overnight or two nights until bacterial suspension becomes turbid.
2. On the second day before cocultivation, add 2 ul of 0.5 M AS to overnight-grown bacteria and mix well. Dilute bacteria and AS suspension ten-fold with LB in a 10-cm glass Petri dish (final concentration of AS = 20 μM). For control treatment, apply 4 ml of LB in a separate Petri dish.
3. Prepare one or two Magenta GA-7 boxes containing etiolated shoots. Use a new #11 scalpel to carefully cut internodes (1 cm) with the help of a pair of forceps. Drop the internode explants immediately into the

diluted *Agrobacterium* suspension. Immerse individual explants into the suspension with forceps. Immerse control explants in LB medium in the same way as *Agrobacterium*-treated one. Time in suspension is about 8 minutes for 40 explants.

4. Transfer all explants of the same treatment side by side closely spaced in the center of a plastic Petri dish containing about 25 ml of solid Cmod medium. Seal dishes with Parafilm. Incubate dishes at 25°C in the dark for 2 to four days.
5. On the third day, examine the cocultured explants. If bacteria layer is visible around the explants, it is time to transfer explants onto selection medium.
6. Pick individual internode explants, remove excess *Agrobacterium* cells by inserting explants into an unused portion of the original cocultivation medium. Transfer 20 to 25 explants onto a 10-cm plastic Petri dish with Cmod medium containing 500 μg/ml carbenicillin or 250 μg/ml cefotaxime and 50 μg/ml kanamycin.
7. Incubate Petri dishes in the dark at 25°C for one to two months. Transfer explants into a fresh selection medium with both antibiotics once a month. Examine at least once a week for any contamination.
8. In the second month, examine cocultured explants to see if any callus formed on the cut ends. If some of the explants form callus, then transfer all to regeneration medium (H1) containing 250 μg/ml carbenicillin or 250 μg/ml cefotaxime and 50 μg/ml kanamycin, and incubate the Petri dishes in weak light (about 4 $\mu Em^{-2}sec^{-1}$, 16 hr light/8 hr dark cycle) at 25°C.

9. One month after incubation of explants in weak light, pale green callus or embryo-like structures will be visible. Transfer these callused explants to a fresh H1 medium with both antibiotics and incubated in the same conditions. If the growth of green calli is very slow, transfer all explants to C medium containing 250 ug/ml carbenicillin and 50 μg/ml kanamycin for one to two months, then transfer back to H1 medium with antibiotics.
10. Approximately 6 to 12 months following cocultivation, green shoots on the calli are removed with forceps or a scalpel and transferred onto H2 medium containing 50 μg/ml kanamycin in Magenta GA-7 boxes. Incubate in higher light (32 $\mu Em^{-2}sec^{-1}$, 16 hours light/8 hours dark cycle) at $25^{o}C$ for one to two months. Change a fresh medium once a month or two months. Discard those shoots with bleached newly developed leaves.
11. During this period, roots emerge from the base of shoots. Transfer putative kanamycin-resistant plantlets or shoots to the same H2 medium, except kanamycin is increased to 75 μg/ml for screening true kanamycin-resistant plantlets.
12. Transfer kanamycin-resistant plantlets onto fresh H2 medium with 75 μg/ml kanamycin, 9 to 16 plantlets per GA-7 box.

Notes:

1. Anthurium cultivars 'Rudolph' and UH1060 can be successfully cocultivated with *Agrobacterium* using this protocol.

2. Kanamycin at 50 μg/ml may be inhibitory to callus formation in the internodes, try to reduce kanamycin concentration to 25 μg/ml. After callus formation, change to higher kanamycin concentration (50 μg/ml).
3. Other explants, such as intact laminae or root segments from in vitro-grown plantlets can also be used for cocultivation. However, C medium (laminae) or H1 medium (both laminae and root segments) should be used for cocultivation, then follow the same protocol for selection and regeneration.

APPENDIX D

COCULTIVATION OF TOBACCO LEAF SEGMENTS WITH *AGROBACTERIUM*

MEDIA AND SOLUTIONS:

1/2MSO (per liter)(root induction medium):

MS macro	50 ml
MS micro	10 ml
MS F(7340 mg/l)	3.5 ml
MS G	5 ml
Myo-inositol	100 mg
Sucrose	30 g
Difco Bacto-Agar	7 g

pH 5.6-5.8

1/2MS104 (per liter)(cocultivation and selection):

MS macro	50 ml
MS micro	10 ml
MS F(7340)	3.5 ml
MS G	5 ml
Myo-inositol	100 mg
BA (0.2 mg/ml)	5 ml
NAA (0.2 mg/ml)	0.5 ml
Sucrose	30 g
Difco Bacto-agar	7 g

pH 5.6-5.8

Acetosyringone (AS) stock: 0.5 M in DMSO

Dissolve 0.25 g of AS in 2.55 ml dimethyl sulfoxide (DMSO)

Filter (0.22 μm) sterilize the solution

Store IN THE FREEZER (-20^{o}C)

GUS reaction buffer:

Sterile distilled water	30 ml
10 mM EDTA·Na_2	0.186 g
100 mM $NaH_2PO_4.H_2O$	0.69 g
0.5 mM $K_4Fe(CN)_6.3H_2O$	0.0105 g
0.1% Triton X-100	0.05 ml

pH 7.0

Add sterile water to 50 ml

Store at 4^{o}C

X-gluc stock:

Dissolve 50 mg X-gluc in 1 ml DMSO

Working solution:

Add 10 μl X-gluc stock soln into 1 ml of GUS reaction buffer

Ref. McCabe, D. E., W. F. Swain, B. J. Martinell & P. Christou. 1988. Stable transformation of soybean (*Glycine max*) by particle acceleration. Biotechnology 6:923-926.

PROTOCOL:

1. Culture a single colony of *Agrobacterium tumefaciens* LBA4404 carrying various plasmids from freshly cultured plates in 5 ml LB plus 50 μg/ml kanamycin and 25 μg/ml streptomycin, 220 rpm, 28°C. This step may take 2 to 3 days.
2. Add 2 μl 0.5 M AS stock to each bacterial culture on the second (or third) day (final concentration 200 μM).
3. Dilute bacterial culture 10-fold with LB (pH 7.0): 0.4 ml of bacteria and AS suspension plus 3.6 ml LB without antibiotics.
4. Cut leaf explants from seedlings (previously grown in 1/2MSO medium) into about 0.5 x 1 cm. The tissues with middle vein and from edges are discarded.
5. Immediately immerse explants in diluted *Agrobacterium* and AS suspension (the final concentration of AS is 20 μM).
6. Place explants in solid 1/2MSO104 for 2-3 days at 25°C in the dark.
7. Transfer leaf explants to MS104 medium containing 500 μg/ml carbenicillin and 300 μg/ml kanamycin in 10-cm plastic Petri dishes. Incubate for several (usually 4) weeks in the light (32 μE m-2sec-1, 16 hr light/8 hr dark cycle) until you can see green shoot initials with a dissecting microscope. At this stage, some calli are also visible.
8. Transfer enlarged leaf explants to the same fresh selection medium.
9. After 2-4 weeks, cut regenerated shoots (about 0.5 cm long) and transfer to 1/2MSO containing 500 μg/ml carbenicillin and 100 μg/ml kanamycin to allow root formation.
10. Cut the shoot tip (with about 2-3 expanded leaves) and inserted into the same fresh rooting medium (1/2MSO) with 100 μg/ml kanamycin. If

bacteria growth occurs, transfer the shoots onto 1/2MSO with carbenicillin (500 μg/ml) and kanamycin (100 μg/ml).

11. Rooted plantlets without sign of bacteria growth can be transfered to 6-inch pots containing commercial potting mix or prepared by yourself (peatmoss: perlite: #4 vermiculite = 1:1:1).

Note: After about two months, when plants begin to flower, you can self-pollinate or backcross with the untransformed wild type plants.

12. For GUS histochemical assay, cut a small piece of leaf (0.5 x 0.5 cm) and staine for GUS expression by incubating in 50-100 μl reaction solution, and incubated at 37^{o}C for 2-24 hours. Add 1 ml of 95% ethanol to clear the chlorophyll if necessary (if the blue color is very weak). You will be able to visualize the blue color by eyes. In case you don't see blue color in leaf samples, try root segments.